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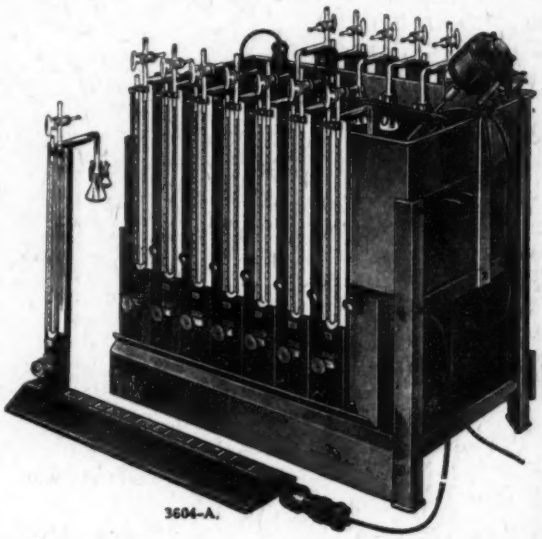

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ENDOGENOUS OXYGEN UPTAKE AND SPECIFICITY OF EMBRYONIC INTRACELLULAR CONSTITUENTS[1]

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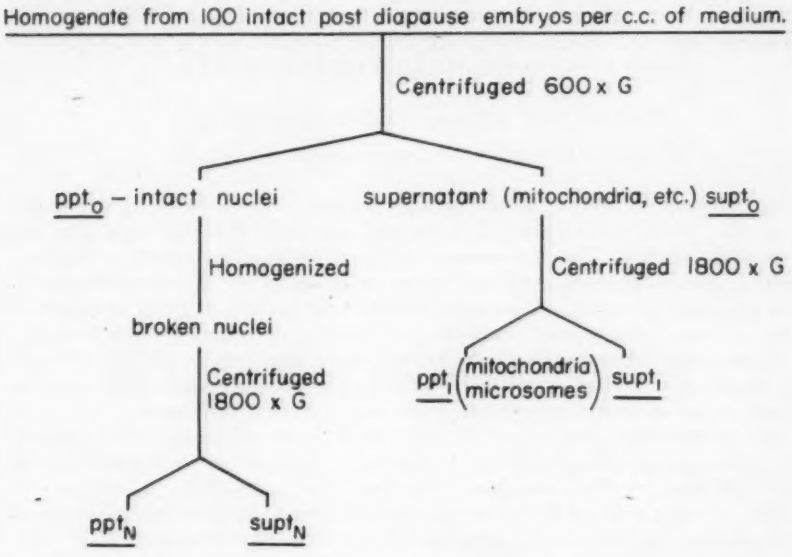
The action and interaction of the various internal parts of the living cell, especially as they relate to its normal functioning, have long been of interest to the experimental biologist. That the reactions of living cells are the results of enzyme-substrate relationships seems well established but many of the details of these phenomena are as yet not clear. The extent to which the nucleus controls or regulates the type or nature of chemical reactions occurring in the cell as a whole has to a great degree been discussed from a theoretical rather than from a scientific point of view (DeRobertis, Nowinski and Saez, 1950). The presence, distribution and localization of intracellular enzyme-substrates and chemical compounds have been variously demonstrated by micro-chemical and other techniques (Caspersson, 1950). The fact that living cells are in themselves able to carry on the reactions essential for their metabolic activities also seems well established. The degree to which the different parts of the cell are specific for these physico-chemical phenomena is at present not too well understood (Caspersson, 1950; Brachet, 1950). Details as to the presence of typical chemical compounds in the different parts of living cells are rapidly accumulating and tend to show a marked regional localization for many of the materials deemed essential for the various functions of the parts of the cell (Lardy, 1949; Caspersson, 1950). Results from many investigations on the enzyme-substrate relations of cellular parts seem to deal largely with systems isolated from the cells and to which various reagents have been added and, conclusions then deduced from results of such extracellular experiments, are referred to the possible conditions in the intact normal cell (Schneider, 1946; Schneider, Claude and Hogeboom, 1948; Lardy, 1949). Valuable data, however, have accumulated from investigations carried out by these methods. It seems reasonable to assume that investigations of the endogenous metabolism of the intact living cell and its parts should contribute additional data to an understanding of intracellular phenomena. To this end, extensive experiments have been carried out on the endogenous oxygen uptake of the various parts of the normal living embryonic cells of the grasshopper embryo. The embryo of the grasshopper (*Melanoplus differentialis*) has many advantages for such investigations since it can be rigidly controlled and standardized (Bodine and Lu, 1950a). Yolk and other extraneous materials

[1] Aided by a grant from the National Institutes of Health. Acknowledgment is gratefully made to Etta Andrews for technical assistance in carrying out these experiments.

which normally interfere to a marked degree in such experimental work can be practically eliminated. Since the embryo is a cold-blooded animal, rigid temperature and developmental controls can easily be maintained (Slifer, 1931). The present data are concerned with the endogenous oxygen uptake of the homogenates and intracellular parts of the embryonic cells of mitotically active (postdiapause) and developing embryos.



supt$_O$ = supernatant from centrifugation of homogenate of embryo – cytoplasm (microsomes, mitochondria, substrate)
ppt$_O$ = precipitate " " " " " " – nuclei (intact).
ppt$_N$ = " " " " " " nuclei – solid constituents of nuclei.
supt$_N$ = supernatant " " " " " " – non-solid constituents of nuclei
ppt$_I$ = precipitate " " " " " cytoplasm – mitochondria, microsomes(?)
supt$_I$ = supernatant " " " " " " – largely substrate (?)

FIGURE 1.

MATERIALS AND METHODS

Embryos of the grasshopper (*Melanoplus differentialis*), dissected from eggs of known developmental and temperature history, have been used throughout, while all methods employed were essentially similar to those already described (Bodine and Lu, 1950a). Oxygen uptake was measured with standard Warburg manometers at 25° C. using flasks of 5 ml. capacity. Micro-differential manometers were used for isolated nuclei and nuclear homogenates. Preparation of homogenates and

fractionation of cellular parts were carried out as previously indicated (see Fig. 1) (Bodine and Lu, 1950a). Cytological examinations, using the phase microscope, were made on all materials used. Ringer's solution, phosphate buffered (pH 6.8), was used as suspension medium. Concentrations of intracellular constituents were so chosen that 1 ml. of medium contained the equivalent of those obtained from 100 embryos of known developmental history. Figure 1 gives a diagramatic outline of procedures as well as symbols for fractions produced.

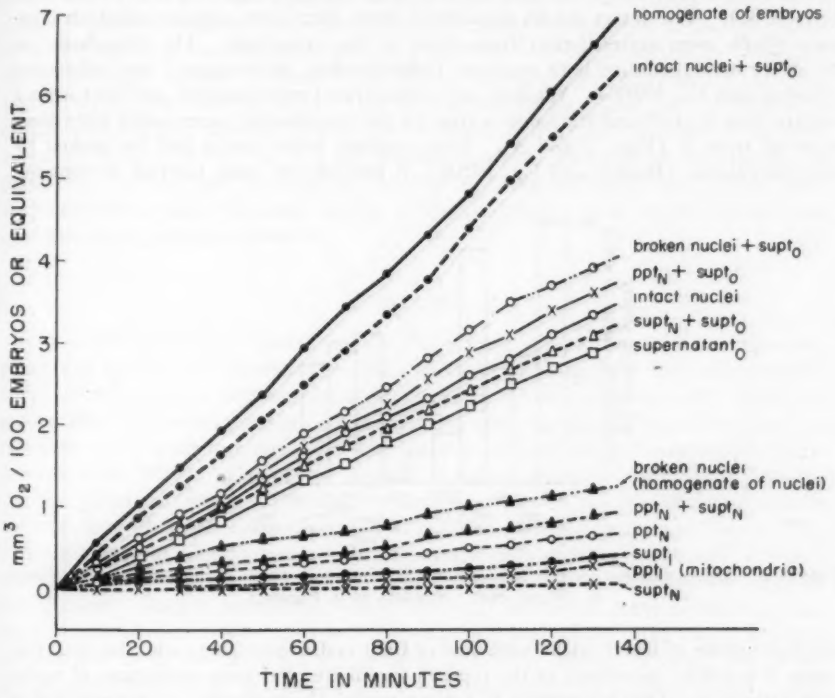


FIGURE 2. Shows oxygen uptake of homogenate of embryo and its constituent parts. Ordinate, mm.^{3} oxygen per 100 embryos or its equivalent. Abscissa, time in minutes. Symbols same as in Figure 1.

RESULTS OF EXPERIMENTS

Inasmuch as results for different lots of embryos are qualitatively similar, only typical cases for eggs of uniform ages and developmental histories will be presented. In general, all embryos and their products thus far tested have given similar results.

The relations between the oxygen uptake of mitotically active (postdiapause) and blocked (diapause) embryos and their homogenates have previously been pointed out (Bodine, 1950). We shall deal here with the endogenous oxygen uptake of embryo homogenates, nuclei, and nuclear homogenates, as well as specific

differences and similarities between enzyme-substrates in the various intracellular parts. Results for typical experiments are graphically shown in Figures 2 and 3.

An examination of Figure 2 shows that the endogenous oxygen uptake of fractionally separated intact nuclei and supernatant (cytoplasm) is approximately 50 per cent of that for the homogenate from which they are derived (Bodine and Lu, 1950a). Upon recombination of the two parts (ppt.$_o$ + Supt.$_o$) oxygen uptake is approximately 90–95 per cent of that for the original homogenate. These results confirm those previously reported for somewhat similar experiments (Bodine and Lu, 1950a). The intact nuclei apparently have their own enzyme-substrate systems which seem quite distinct from those of the cytoplasm. The cytoplasm, on the other hand, contains both enzymes (mitochondria, microsomes) and substrates (Bodine and Lu, 1950b). Washed and concentrated mitochondria and microsomes respire little if at all and the same is true for the cytoplasmic supernatant after their removal from it (Figs. 2 and 3). Since washed intact nuclei can be broken by homogenization (Bodine and Lu, 1950b), it becomes of some interest to compare

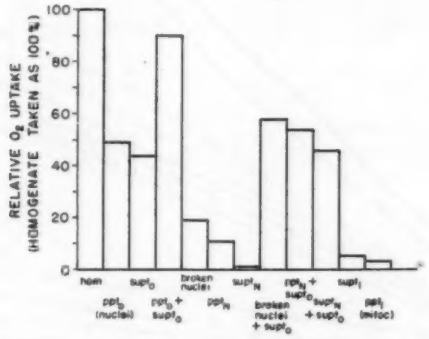


FIGURE 3. Shows relative oxygen uptake for 120 minutes in terms of homogenate of embryo as 100 per cent. Symbols as in Figure 1.

oxygen uptake of intact nuclei with that of their constituent parts and also to determine, if possible, something of the relative specificity of enzyme-substrates of nuclei and cytoplasm. Oxygen uptake of broken nuclei (homogenate) is reduced below that of the intact ones and falls to approximately 30–35 per cent of the original value (Figs. 2 and 3). The broken nuclei are readily separated by fractional centrifugation into solid particles and a liquid supernatant. The oxygen uptake of the supernatant nuclear fluid is practically zero while the solid fraction, even though washed, respires at approximately 50 per cent of the nuclear homogenate rate (Figs. 2 and 3). By recombining the nuclear fragments with the fluid supernatant, a close approximation to the original oxygen uptake is obtained, similar to the case of recombinations of the constituent parts of embryonic homogenate (Bodine and Lu, 1950b).

By making suitable combinations of nuclear and cytoplasmic fractions, rather striking evidence for a marked degree of specificity in the endogenous oxygen enzyme-substrate systems becomes apparent. Broken nuclei (or homogenates of

nuclei) added to cytoplasm produce a greater oxygen uptake than cytoplasmic or nuclear homogenates alone and the sum total of the oxygen uptake is approximately equal to the sum of that for the two systems (Fig. 3). Nuclear supernatant added to cytoplasm produces no significant change in the oxygen uptake over that for cytoplasm alone. The solid particles of the nuclei added to cytoplasm produce an oxygen uptake equal to that of the sum of the two constituent fractions. It seems reasonable to assume, therefore, that the enzyme activity of the nucleus is confined to the solid parts and that substrate resides largely in the fluid parts. Since cytoplasm produces no marked increases in oxygen uptake when added to nuclear fluids, it would seem that mitochondria and other enzyme-bearing structures in cytoplasm do not act upon nuclear substrates and that a degree of specificity exists for cytoplasm and nucleus as regards this type of reaction (Figs. 2 and 3). Inasmuch as endogenous oxygen consumption is that confined *entirely* to the inherent enzyme-substrate systems of the cell, it is not possible from the above results to separate or point out specific types of enzymes involved in these reactions, but it does seem reasonable to assume that some degree of specificity does exist for the nuclear and cytoplasmic respiration systems.

Discussion

The extent to which a specificity for the enzyme-substrate systems of the cytoplasm and nucleus can be demonstrated should throw some light upon the probable chemical interchanges between these structures in the normal intact cell. Evidence gained from the localization of certain chemical compounds like the nucleic acid derivatives would seem to indicate but little passage of materials between the two parts (Caspersson, 1950). That some substances must constantly interchange between the two structures seems reasonable to suppose in view of the equilibrium normally existing between them (Caspersson, 1950; DeRobertis, Nowinski and Saez, 1950). From the present observations on the embryonic cells of the grasshopper, it would seem that for the endogenous oxygen uptake studied, the two systems are more or less distinct. Such a postulation would hold only for those enzyme-substrate systems connected with the endogenous oxygen uptake and would in no way contribute information as to the parts played by other systems. Since the living cell of the grasshopper embryo is stimulated by 2,4-dinitrophenol only when *intact*, while methylene blue stimulates oxygen uptake almost solely by its action on the intact nucleus, one might reasonably assume from such reactions a marked difference in the physico-chemical workings of the various parts of the cell (Bodine and Lu, 1950c). Evidence recently acquired shows that succinate added to homogenates of embryo increases oxygen uptake while no effect is noted when intact or homogenized nuclei are employed. Structural or morphological relations and "intactness" of cells undoubtedly contribute much to the rates at which various localized enzyme-substrate systems work. Localization of substrates in the intact cell is greatly changed or disturbed in homogenates. However, in the case of the embryo, isolated intact nuclei—when combined with cytoplasm—show oxygen uptake of approximately 90–95 per cent of that found for the homogenate from which the constituents were taken (Bodine and Lu, 1950b).

SUMMARY

1. The endogenous oxygen uptake of the homogenate of the embryo of the grasshopper, *Melanoplus differentialis*, along with the intracellular constituents of the embryonic cells has been measured.

2. The endogenous oxygen uptake of intact washed nuclei and their homogenate as well as that of the intranuclear constituents has also been measured.

3. Respiratory enzymes of the nucleus are located in the solid particles obtained from fractional centrifugation.

4. Enzymes of cytoplasm and nucleus appear specific since no change in oxygen uptake occurs when they are added to reciprocal substrates.

5. Fractions of homogenates of embryo or nuclei when recombined have oxygen uptakes of approximately 90–95 per cent of the original homogenates.

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CYTOCHROME OXIDASE AND SUCCINIC DEHYDROGENASE CONTENT OF SQUID (LOLIGO PEALII) NERVOUS TISSUE

S. J. COOPERSTEIN AND ARNOLD LAZAROW

Western Reserve University School of Medicine and Marine Biological Laboratory, Woods Hole, Mass.

In the course of investigations designed to elucidate the metabolic differences between normal and chromatolytic nervous tissue, the distribution of succinic dehydrogenase and cytochrome oxidase was studied in various parts of the nervous system of the dog. We found that whereas white matter and spinal nerve had measurable quantities of cytochrome oxidase, both of these tissues contained less succinic dehydrogenase than could be detected by the methods used (Cooperstein, Lazarow and Kurfess, 1950; Schneider and Potter, 1943). In contrast, grey matter and spinal ganglia contain measurable quantities of both of these enzymes and the ratio of cytochrome oxidase to succinic dehydrogenase was found to be 3.9 and 3.2 respectively. If this same enzyme ratio were also present in white matter (or in nerve), the succinic dehydrogenase would have been easily detected. Conversely, considering the sensitivity of the succinic dehydrogenase method employed, it is safe to say that the cytochrome oxidase: succinic dehydrogenase ratio is at least 19.0 in white matter and 9.0 in spinal nerve (Cooperstein, 1951).

Since cytochrome oxidase and succinic dehydrogenase are almost invariably present together in tissues, this dissociation of enzyme activity in nerve and white matter warrants further investigation. It therefore seemed advisable to determine the enzyme content in samples of nerve axoplasm which are not contaminated with the myelin sheath. This can be accomplished in the squid (*Loligo pealii*), where the giant axone can be dissected and samples of pure axoplasm can be obtained in amounts sufficient for microanalyses.

Nachmanson *et al.* (Nachmanson and Meyerhof, 1941; Nachmanson and Steinbach, 1942; Nachmanson, Steinbach, Machado and Spiegelman, 1943) previously studied the distribution of a number of enzymes in squid nervous tissue and found that whereas cholinesterase was more concentrated in the nerve sheath, succinic dehydrogenase and cytochrome oxidase were more concentrated in the axoplasm. Since they used macro-analytical methods, combined samples obtained from different axones were required for each determination. Therefore, relatively few determinations were carried out, and some of the activities reported were at the lower limits of the sensitivity of the methods employed. Furthermore, the assay methods used are less dependable than some of the more recently developed techniques (see Discussion). It therefore seemed advisable to repeat this study using micro-methods (Cooperstein, Lazarow and Kurfess, 1950; Cooperstein and Lazarow, 1951).

Experimental

Methods

Dissection: Squid (*Loligo pealii*) were decapitated and the mantle opened along the mid-ventral line. The bilateral nerve trunks originating from each stel-

late ganglion and containing the giant axone were identified. The giant axone was dissected *in situ* and freed of the extraneous connective tissue sheath and of the small axones which surround the giant axone. The axoplasm is surrounded by a single layer of cells, the inner protoplasmic sheath (which corresponds to the vertebrate Schwann cells), and external to this there is a metatropic sheath which is lipoid-containing. This metatropic sheath, which is similar to the vertebrate myelin sheath except that it lies outside the protoplasmic cell layer, is a few microns thick and can be detected in polarized light (Bear, Schmitt and Young, 1937). It blends with the innermost layers of the outer connective tissue sheath which surrounds the axone, and it usually remains attached to the cleaned giant axone. The right cleaned giant axone with surrounding metatropic sheath was removed. A sample of the fibrous connective tissue sheath (plus the many small axones) which surrounds the giant axone was also taken. The left giant axone was similarly dissected, tied at two places (to prevent loss of axoplasm), removed, and placed on a sheet of Parafilm. One of the ligatures was then removed and a sample of axoplasm obtained by squeezing the nerve between two sheets of Parafilm. Pieces of fin nerve, stellate ganglion, and muscle were also taken. The various samples of tissue weighing between 2 and 10 mg. were placed between two folds of a Parafilm sheet and weighed to 1/100 of a milligram on a Roller-Smith Torsion Balance.[1] The tissue was then transferred to a micro conical-tipped glass homogenizer and kept at 0° C. The Parafilm sheets were then re-weighed. One axone yielded sufficient axoplasm for one set of determinations.

Enzyme determinations: In the succinc dehydrogenase assay (Cooperstein, Lazarow and Kurfess, 1950), the tissues were homogenized at 0° C. in a succinate-phosphate solution (0.5 *M* sodium succinate in 0.2 *M* phosphate buffer, pH 7.4) and diluted with the same solution. The results are expressed as "Standard Enzyme Activity," which is calculated as Δ log [ferricytochrome *c*]/minute for a final tissue dilution of 1 : 100.[2]

In the cytochrome oxidase assay (Cooperstein and Lazarow, 1951), the tissues were homogenized at 0° C. and diluted with 0.033 *M* phosphate buffer, pH 7.4. The results are expressed as "Standard Enzyme Activity," which is calculated as Δ log [ferrocytochrome *c*]/min. for a final tissue dilution of 1 : 100.[3] Two determinations were run on each sample of tissue. In the succinic dehydrogenase assay, 10 μl. and 20 μl. of an appropriate dilution were tested; in the case of the cytochrome oxidase, 2 μl. and 4 μl. were used.

[1] The Parafilm is obtainable from the Marathon Corporation, Menasha, Wisconsin. Suitable weighing papers measuring 10 × 20 millimeters, and weighing between 5 and 10 milligrams are made by stretching the Parafilm sheet at right angles to roll. Tissues enclosed between two folds of Parafilm do not dry out. The Roller-Smith Balance has a total capacity of 25 mg.

[2] Example: When 10 μl. of a 1 : 60 dilution of stellate ganglion were added to 290 μl. of a cytochrome *c*-cyanide solution (final dilution = 1 : 1800) and the logarithms of the extinctions at 550 mμ (corrected for the blank) were plotted against time, the Δ log [cytochrome c^{+++}]/minute was found to be 0.37. This gave a

$$\text{standard enzyme activity} = \frac{\Delta\log[\text{cyto. } c^{+++}]/\text{minute} \times \text{tissue dilution} \times 300}{\text{volume of enzyme added for test} \times 100}$$

$$= \frac{.037 \times 60 \times 300}{10 \times 100} = 0.666.$$

[3] Calculated as in footnote 2.

Results

Succinic dehydrogenase: The succinic dehydrogenase contents of the various tissues are shown in Table I. Stellate ganglion was found to have a significantly

TABLE I

Distribution of succinic dehydrogenase in squid tissues

Tissue	Final dilution tested	No. determinations	Average standard enzyme activity (1:100 tissue dilution)	σ*	P† with respect to next lower
Stellate ganglion	1:1800	8	0.682	0.081	< .001
Muscle	1:1200	7	0.466	0.051	< .001
Cleaned giant axone	1:900	6	0.360	0.033	>0.1
Fin nerve	1:900	5	0.340	0.072	>0.1
Fibrous sheath + small nerves	1:900	7	0.337	0.063	>0.1
Axoplasm	1:900	7	0.324	0.060	

* Standard deviation $= \sqrt{\frac{\Sigma(\text{deviations from mean})^2}{N}}$.

† P value of $\frac{\text{Difference}}{\sigma\ \text{Difference}}$.

higher content of this enzyme than muscle. Although the succinic dehydrogenase contents of the giant axone, fin nerve, fibrous sheath plus small nerves, and axoplasm do not differ significantly from each other, they are all less active than muscle.

TABLE II

Distribution of cytochrome oxidase in squid tissues

Tissue	Final dilution tested	No. determinations	Average standard enzyme activity (1:100 tissue dilution)	σ*	P† with respect to next lower
Stellate ganglion	1:15,000	8	6.46	0.827	< .001
Muscle	1:12,000	8	4.60	0.615	< .001
Fibrous sheath + small nerves	1:3,000	8	1.23	0.090	>0.1
Axoplasm	1:3,000	8	1.19	0.012	>0.1
Cleaned giant axone	1:3,000	7	1.12	0.210	>0.1
Fin nerve	1:3,000	4	1.08	0.105	

* Standard deviation $= \sqrt{\frac{\Sigma(\text{deviation from mean})^2}{N}}$.

† P value of $\frac{\text{Difference}}{\sigma\ \text{Difference}}$.

Cytochrome oxidase: Table II shows the distribution of cytochrome oxidase. The tissues were found to have the same relative order of activity with respect to cytochrome oxidase as they do with respect to succinic dehydrogenase.

Ratio cytochrome oxidase/succinic dehydrogenase: Comparing the ratios of cytochrome oxidase to succinic dehydrogenase for the various tissues, it was found that those tissues with the highest enzyme activities also have the highest oxidase to dehydrogenase ratio (ganglion, 9.48; muscle, 9.88), whereas those tissues with the lowest enzyme activities also have the lowest oxidase to dehydrogenase ratio (cleaned giant axone 3.12; axoplasm, 3.67; fibrous sheath, plus small nerves, 3.65; fin nerve, 3.48).

Discussion

Our results do not agree quantitatively with those obtained by Nachmanson *et al.* (Nachmanson and Steinbach, 1942; Nachmanson, Steinbach, Machado and Spiegelman, 1943) in that we did not find succinic dehydrogenase or cytochrome oxidase to be concentrated in the axoplasm. (However, these findings do not alter the primary conclusions of these authors in that the majority of the succinic dehydrogenase and the cytochrome oxidase of squid nerve is present in the axoplasm since the major fraction of the giant axone is axoplasm.) We found that the fibrous connective tissue sheath (plus small nerves) surrounding the giant axone contains as much enzyme as does the axoplasm. We did not determine the enzyme contents of the axonal membrane plus metatropic sheath (residual tissue after squeezing out the cleaned axoplasm) because it is difficult to get sufficient material for analysis. For example, a cleaned giant axone which gave 2.07 mg. of axoplasm yielded only 0.25 mg. of residual tissue (after extrusion of axoplasm).

It is possible that part of the apparent enzyme concentration which they reported for axoplasm may have been due to drying. In our studies we have taken precautions to prevent drying by enclosing the pieces of tissue between two sheets of Parafilm (the weight loss under these conditions is less than 0.05 mg. in two hours), and less than 15 minutes elapsed between removal of the axone and its homogenization.

While the discrepancy in the results is not completely explainable, our methods are probably more reliable than those used by Nachmanson *et al.* Since they report that the respiratory rate of these tissues decreases progressively with time after removal from the animal, the assay methods which we have employed offer the advantage that much less time is required for completion.

In the case of the cytochrome oxidase assay, they used p-phenylenediamine as a substrate. This method has certain drawbacks which interfere with its accuracy (Rosenthal, 1937; Stotz and Hastings, 1937), and these errors are magnified at low oxygen uptakes. Another factor to consider is the possible toxic action of some of the end products of p-phenylenediamine oxidation (Rosenthal, 1937). The method which we used for cytochrome oxidase is not subject to these limitations since cytochrome *c* itself is employed as a substrate.

Their succinic dehydrogenase determinations were carried out in two ways. In the first, the oxygen uptake was measured in the presence of succinate and cytochrome *c*, the activity being a measure of the complete succinoxidase system which includes cytochrome oxidase, cytochrome *c*, succinic dehydrogenase, and probably other unknown factors. The second method utilizes ferricyanide as a direct electron acceptor from succinic dehydrogenase without the intervention of the cytochromes. Although Nachmanson *et al.* report good agreement for the rate of utilization of

succinate as determined by these two methods, they made their calculations on the assumption that oxygen is reduced to hydrogen peroxide by the complete succinoxidase system. If, however, one recalculates the data on the basis that water is the end product, and if the oxidation does not proceed further than fumarate or malate, views which are more generally accepted (Hogness, 1939; LuValle and Goddard, 1948; Keilin and Hartree, 1940), their two methods do not agree and one reaches the conclusion that the rate of oxidation of succinate by the complete succinoxidase system is twice as rapid as its rate of oxidation by one of the components of the succinoxidase system, *i.e.*, succinic dehydrogenase. This is an obvious impossibility, since the rate of oxidation by the complete system can be no faster than the turnover rate of its slowest component. Thus, the turnover rate of the dehydrogenase as measured in the ferricyanide test must be considerably lower than in the natural succinoxidase system. Similar inhibition by another artificial carrier (methylene blue) used in a test for succinic dehydrogenase has also been observed (Potter, 1941; Cooperstein, Lazarow and Kurfess, 1950).[4] The method which we used for the determination of succinic dehydrogenase has the advantage over the ferricyanide test in that the natural substrate, *i.e.*, oxidized cytochrome *c* is used. The advantages of this assay method over that using the complete succinoxidase system as a test for succinic dehydrogenase have been previously discussed.

The enzyme dissociation previously observed in the white matter and spinal nerve of the dog has not been found in squid nerve or samples of squid axoplasm. In fact, there is a lower ratio of cytochrome oxidase: succinic dehydrogenase in squid axoplasm and sheaths than there is in squid stellate ganglion or muscle. Therefore, the low succinic dehydrogenase content observed in dog nerve is not a general phenomenon. It is possible that the large amount of myelin present in this species somehow interferes with the determination of succinic dehydrogenase. However, since the addition of nerve or white matter to other tissues does not result in any inhibition of their succinic dehydrogenase activity, this explanation would not appear to account for the results.

Summary

1. The cytochrome oxidase and succinic dehydrogenase contents of various squid tissues, including giant axone and samples of axoplasm, have been determined and compared with previous findings in dogs. In the case of both enzymes studied, the stellate ganglion of the squid is approximately 1.5 times as active as squid muscle. Squid ganglion has approximately six times as much cytochrome oxidase as the other nervous tissues examined, but only about two times as much succinic dehydrogenase. Our findings, in contrast to previously reported results, reveal no evidence of concentration of these enzymes in axoplasm.

2. The markedly low (or absent) succinic dehydrogenase content which was found in dog nerve and white matter was not observed in the case of the squid nerve, axoplasm, or sheaths.

[4] It has been further reported that a ferricyanide concentration of approximately 1/50th that used in the assay method for succinic dehydrogenese causes a 28 per cent inhibition of the oxygen uptake of the complete succinoxidase system (Barron and Singer, 1945). We have found that a concentration of ferricyanide equal to that used in the assay method causes a 91 per cent inhibition of rat brain succinoxidase.

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PHOSPHATASE ACTIVITY IN TETRAHYMENA[1]

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Histochemical studies by Weisz (1949) with normal and re-organizing Stentors and those by Sullivan (1950) with *Colpidium campylum* have demonstrated the presence of phosphatases in Protozoa. The present investigation was undertaken to increase our knowledge concerning the action of these enzymes in the ciliate protozoan, *Tetrahymena geleii*, and to correlate this activity with growth.

Materials and Methods

The organism employed in this investigation, strain E, was maintained in 250 ml. Erlenmeyer flasks under aseptic conditions except where sterility was not necessary. The media were prepared with double glass-distilled water and were sterilized in the autoclave 10 minutes at 15 pounds pressure. The pH was adjusted to 7.4 with 1/*N* NaOH before sterilization except where pH was specifically under investigation. All cultures were incubated at 24° in the dark. The numbers of organisms were estimated with the Klett-Summerson photoelectric colorimeter according to a method reported earlier (Elliott, 1949b).

Analyses for total and inorganic phosphorus were performed with the methods of Fiske and Subbarow (1925). At stated intervals 6-ml. samples were withdrawn aseptically with pipettes and the organisms separated from the supernatant by centrifugation (100 times gravity for 2 minutes). Phosphorus determinations were made on 2-ml. aliquots of the supernatant. The organisms were washed twice and then re-suspended in distilled water for estimating numbers in the colorimeter. Finally, where indicated, total phosphorus determinations were made on 2-ml. aliquots of the organisms. All experiments were repeated several times and the most representative selected for reporting here.

Experimental

Presence of phosphatases

In order to demonstrate the presence of phosphatases (nucleotidases) in Tetrahymena, a heavy suspension of the cells (approximately 20,000 cells/ml.), taken from proteose-peptone medium during the logarithmic growth phase, was washed twice in distilled water and then suspended in a 0.0005 *M* solution of the nucleotide,

[1] This investigation was supported in part by a grant from the Horace H. Rackham School of Graduate Studies. The authors are grateful to Merck and Company for supplying the amino acids and vitamins used in this investigation, and to Dr. E. L. R. Stockstad of the Lederle Laboratories for furnishing the protogen, without which these experiments could not have been carried out.

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adenylic acid. For convenience the rigid sterile technic used in the remainder of the experiments was somewhat relaxed because it was felt that the combined effects of deficient medium and bacterial predation by the ciliate were adequate to control the effect caused by any slight contamination that might occur. The pH was adjusted electrometrically (Beckman) to 7.4 with 1/*N* NaOH. Final pH determinations indicated a slight drop, but not below 7.1. Inorganic phosphorus determinations were made at intervals for 50 hours of incubation and are recorded in Figure 1.

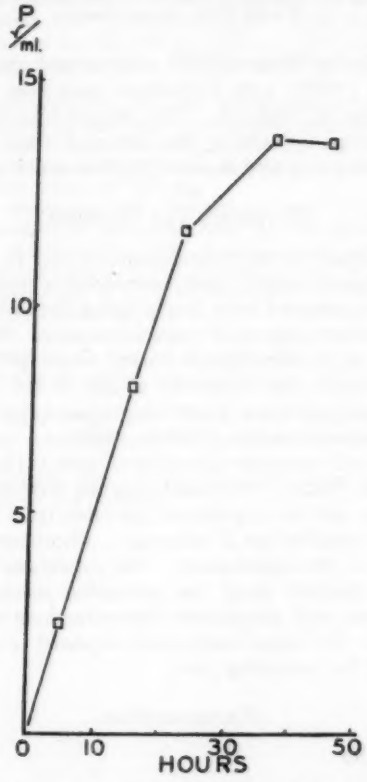

FIGURE 1. Amount of phosphate released plotted against time.

Over 80 per cent of the phosphate in adenylic acid was split off in the first 22 hours and the remaining 20 per cent was released in the next 13 hours. Parallel experiments with cytidylic acid demonstrated similar phosphate release, thus indicating the presence of active nucleotidases.

Location of the enzymes

The question as to whether the enzymes were confined to the cells or released into the surrounding medium was solved by repeating the above experiment, except

that after 14 hours of incubation one-half of the culture was filtered through a Seitz in order to remove the cells from that half; the amount of phosphate release was followed from that time in both flasks. The results are recorded in Figure 2. It is clear that as soon as the cells were removed from the culture, enzyme activity ceased. The enzymes are, therefore, confined to the cells and are not perceptibly released into the culture medium.

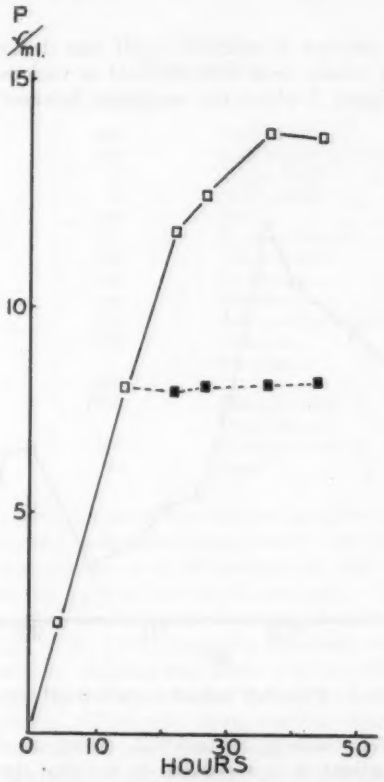

FIGURE 2. Same as Figure 1, except the flask was divided after 14 hours of incubation. Phosphate release indicated by black blocks.

Whether or not the nucleotide was taken into the cell and the phosphate released after being split intracellularly was the next point of interest. If the fate of the adenosine, which results from splitting phosphate from adenylic acid, could be followed, a still more precise location of the enzyme could be predicted. In order to check this point adenylic acid was again prepared as in the two preceding experiments, but this time the absorption of adenine at 260 mμ was followed through 48 hours of incubation, using a Beckman ultraviolet spectrophotometer. Five-ml. samples were removed from the flask at 6–12 hour intervals and the organisms

separated by centrifugation. Appropriate dilutions of the supernatant were made with $N/20$ NaOH and absorption determinations taken.[3] The results of this study indicated that the adenine level remained constant throughout the period of nucleotide breakdown. Apparently, then, the enzyme action takes place either within the cell or at the membrane and perhaps the value of this reaction is to obtain energy for cellular metabolism.

Relation to pH

The activity of the enzymes in relation to pH was demonstrated by following the inorganic phosphate release from adenylic acid at various pH levels. The results are recorded in Figure 3 where the organisms released the greatest amount

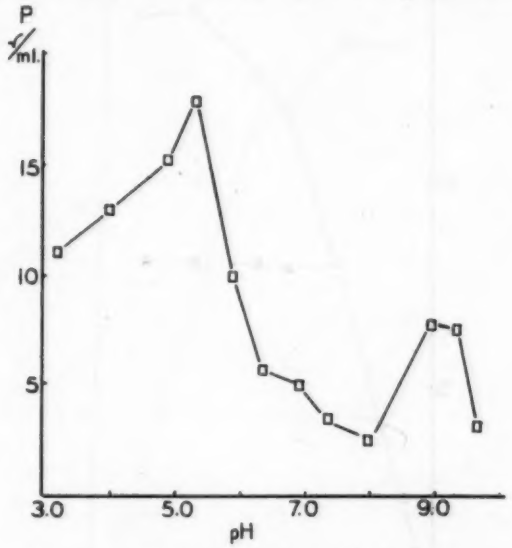

FIGURE 3. Phosphate release at various pH levels.

of phosphate at two levels, namely, 5.3 and 9.2. Since the system is complicated by intact cells in the medium, it is impossible to say that the nucleotidase activity is influenced by pH in the manner shown by these experiments, although it may be.

It might seem strange that all of the experiments were maintained at pH 7.4 rather than at the levels where enzyme activity in this system was optimal. It so happens that both of these levels are outside the range for optimal growth, and therefore might be injurious to the organism. There seems to be no correlation between pH levels which promote optimal phosphatase activity in living cells and those that support optimal growth of those cells.

[3] The adenine determinations were made through the courtesy of Dr. James E. Hogg, Department of Biological Chemistry, University of Michigan. The absorptions of adenine, adenylic acid and adenosine do not differ more than 3 per cent in $N/20$ NaOH solution (Morton, 1942).

Relation to growth

In order to determine phosphatase activity during the logarithmic growth phase of Tetrahymena, it was decided to follow both the release of inorganic phosphate and the phosphate uptake, as indicated by making total phosphorus determinations on both the supernatant and the cells themselves during the logarithmic growth period. The basal medium (Table I) was the same as that employed earlier

TABLE I

Basal medium

	Micrograms per milliliter		Micrograms per milliliter
l-Arginine	600	$FeCl_3 \cdot 6H_2O$	1
l-Histidine	150	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	5
dl-Isoleucine	75	$CuCl_2 \cdot 2H_2O$	5
l-Leucine	75	$MnCl_2 \cdot 4H_2O$	.05
l-Lysine	150	$ZnCl_2$	.05
dl-Methionine	150	Ca pantothenate	.10
dl-Phenylalanine	150	Nicotinamide	.10
dl-Serine	300	Pyridoxine	2.00
dl-Threonine	300	Riboflavin	0.10
l-Tryptophan	150	Pteroyltriglutamic acid	.01
dl-Valine	150	Thiamine	1.0
		Protogen	1.0 unit
Dextrose	1000		
Sodium acetate	1000	Adenylic acid	75
		Guanylic acid	75
$MgSO_4 \cdot 7H_2O$	100	Cytidylic acid	75
$CaCl_2 \cdot 2H_2O$	50	Uracil	75

(Elliott, 1949a, 1950) with the exception that no inorganic phosphate was included in the form of K_2HPO_4 and nucleic acid components were employed instead of yeast nucleic acid. The concentration of these components was raised from 100 to 300 γ/ml., which increased the growth rate by 50 per cent. The only source of inorganic phosphorus was that inadvertently introduced with protogen, an essential growth factor (Stokstad *et al.*, 1949), and this amounted to 2 γ/ml. Six-ml. samples were removed from the experimental flasks and the cells separated by centrifugation; 2 ml. of the supernatant was checked for inorganic phosphorus and another 2 ml. for total phosphorus. The cells were washed twice, then re-suspended in distilled water and numbers estimated in the colorimeter. A 2-ml. aliquot of this suspension was then taken for total phosphorus determinations of the organisms. From these data the curves in Figure 4 were constructed.

The total phosphorus in the supernatant declined at a rate which was the reciprocal of that taken up by the cells, as one might expect. These determinations account for about 95 per cent of the phosphorus. There is an indication that the total supernatant phosphorus continued to decline and the total phosphorus in the cells continued to increase following the growth peak; the reason for this is not clear. It may be that the cells store phosphate after they cease dividing.

The inorganic phosphorus showed the same explosive increase that was noted earlier. Approximately 75 per cent of the inorganic phosphorus is split off while the organisms are still in the lag phase of growth. Once the organisms enter the loga-

rithmic growth phase phosphate is utilized rapidly, hence the curve then shows a decline in the amount of inorganic phosphorus in the medium.

Discussion

Weisz (1949, p. 110), employing histochemical methods, was able to show acid phosphatases in normal Stentors "around the macronuclei, the basal granules of the

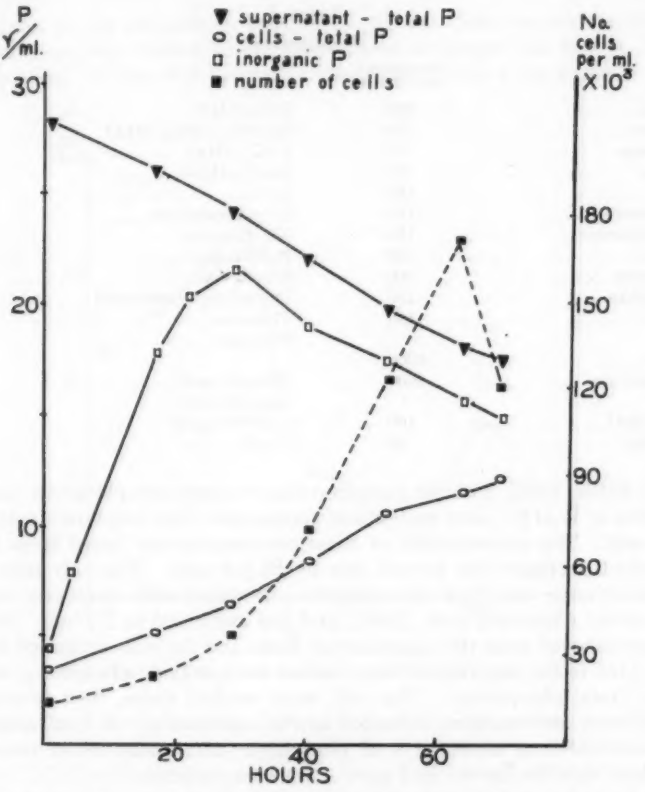

Figure 4. Relation of growth, phosphate release and phosphate uptake.

membranelles and the body cilia, in the endoplasmic fat vacuoles, and around the gastrioles." Sullivan (1950, p. 270), working with similar technics, localized alkaline phosphatase activity "in the perinuclear region of the cell." He used glycerophosphate as a substrate, which may mean that he is not dealing with the same enzymes that are evident in the present investigation. The present observations demonstrate the existence of nucleotidases in *T. geleii* (strain E), but their exact location within the cell cannot be determined from the data. If we are dealing with

the same enzymes as those found by Sullivan, one would expect that the nucleotide (adenylic acid) is absorbed into the perinuclear region of the cell where degradation takes place. This would also mean that the degraded fragments, inorganic phosphate and adenosine, would then be excreted into the medium. However, the present data on both inorganic phosphate and absorption determinations do not demonstrate the absorption of adenylic acid into the cell, only that the molecule is hydrolyzed which may take place at the membrane or intracellularly.

These observations indicate clearly that the enzymes are confined to the organism and are not elaborated into the surrounding medium, which might lend weight to the argument that the enzymes are intracellular. Tetrahymena does produce extracellular enzymes, however, as indicated by its ability to hydrolyze gelatin and casein (Elliott, 1933).

The active nature of these phosphatases in Tetrahymena may have some significance in its natural environment. This organism normally lives at the bottom of ponds where the organic material is undergoing rapid decomposition and where the concentration of inorganic phosphate is high. Numerous studies have shown that the source of this phosphate is in the thin layer of mud at the bottom of the pond or lake where there is a veritable microcosm of flora, fauna and other microorganisms (Einsele, 1938). Perhaps Tetrahymena, along with other microorganisms, aids in splitting off inorganic phosphate from organic debris and helps maintain the phosphate level in these bodies of water.

Summary

1. *Tetrahymena geleii E* possesses phosphatases (nucleotidases) capable of hydrolyzing nucleic acid and its components at a very rapid rate. Eighty per cent of the phosphate in 0.0005 *M* adenylic acid is released in 22 hours when the population level is 20,000 cells/ml.

2. The enzymes are confined to the cells and are not released into the surrounding medium.

3. The cells release phosphate most rapidly at two pH levels, 5.3 and 9.2, being most active in the acid range.

4. Enzyme activity as indicated by phosphate release is greatest in the lag phase of the growth cycle. During the logarithmic growth phase, the released phosphate is taken up by the cells at a uniform rate.

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THE TIDAL RHYTHM OF THE DIATOM HANTZSCHIA AMPHIOXYS

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Several minute chlorophyll-bearing organisms, which inhabit the sand or the mud of the intertidal zone at high tide, migrate to the surface and show photosynthetic activity induced by the sunlight when the tide is low and the beach exposed. At that time they appear on the beach as irregular green, yellow or brown spots.

This behavior is characteristic not only of the well known metazoan *Convoluta roscoffensis,* but also of certain of the Protophyta. Among these are found the Dinoflagellates of the genera Amphidinium, Gymnodinium, Polykrikos (W. A. Herdman, 1911; Storrow, 1913; Whitehead, 1914; Jorgensen, 1918; E. C. Herdman, 1921; Kofoid and Swezy, 1921; Lebour, 1925), the green flagellates of the genus Euglena (Gard, 1919, 1920; Bracher, 1919, 1929; Carter, 1933; Conrad, 1940), the Chrysomonadian *Chromulina psammobia* (Fauré-Fremiet, 1950b), and several diatoms (Bohn, 1904a, 1904b; Fauvel, 1907; Fauvel and Bohn, 1907).

Under natural conditions, the superficial migration of these protophytic organisms appears to be an expression of an intrinsic rhythm which is synchronized with the tide, and which is complicated by the response to light. This rhythm has been studied experimentally in the laboratory, using the following species: the diatom *Pleurosigma estuari* Smith (Fauvel and Bohn, 1907); the green flagellate *Euglena limosa* Gard (Gard, 1919, 1920; Bracher, 1919, 1929, 1938); the Chrysomonadin *Chromulina psammobia* (Fauré-Fremiet, 1950b).

During the past summer a similar behavior was observed in a population of the diatom *Hantzschia amphioxys* Ehrb.,[1] which was found inhabiting the sand of the beach at Barnstable Harbor, Cape Cod.

During the low tide of mid-day in August, irregular spots of different size were visible on the sandy flat near the experimental park of the Woods Hole Oceanographic Institution at Barnstable Harbor. The surface sand, which is slightly muddy, was removed with a spoon and brought to the laboratory for examination. It was then spread over the bottom of large finger bowls and covered with sea water. These cultures were then exposed to the diffuse light from the window.

On succeeding days (the longest period of observation being 6 days), greenish spots similar to those described above re-appeared on the surface of the sand at the time of the low tide at Barnstable Harbor. These disappeared after two to three hours. A microscopic examination showed that the color was due to the superficial accumulation of a great number of the diatom *Hantzschia amphioxys,* associated with a much less numerous undetermined Gymnodinium, and a Euglena.[2]

Hantzschia amphioxys is of rectangular shape, and is slightly asymmetric, having one face convex and the opposite concave. It varies in size from 60 to 80 micra

[1] I wish to express my thanks to Dr. Paul Conger for the determination of this species.

[2] This Euglena resembles *E. limosa* Gard in the lack of flagella and in the metabolic motion, but differs from that form in possessing small and numerous chromoplasts.

in length, and shows two transverse and radiate chromoplasts which are greenish yellow in color. The sliding motion of this diatom is relatively rapid; they travel in the neighborhood of 400 micra in 10 seconds, or about half their total length in one second.

Individuals isolated from the greenish mass exhibit a positive phototropism. When observed under the microscope in a drop of sea water, they move rapidly to the lighted side. This would explain the superficial dispersion of the diatoms both under natural and experimental conditions.

After a few hours, a similar test shows that the phototropistic sign is reversed, the response then being negative. This being true, it would be expected that the diatoms, in the natural habitat, would move into the shadow of the sand grains, leading to a dissolution of the colored spots. The diatoms then have invaded the deeper sandy layers.

During this photophobic phase, the Hantzschia agglutinate among the sand grains. This may well explain why the rising tide does not wash the diatom population away, and has implications of a great general ecological interest.

The observations on the diatoms made in the laboratory with unwashed sand do not permit an exact appreciation of the end-points in the tidal rhythm. Due to the pale color of *Hantzschia amphioxys*, initial phases in surface accumulation and spreading are difficult if not impossible to recognize, and the same is true of the final stages in the disappearance of the surface masses. The behavior of this diatom presents, therefore, a statistical aspect.

It is certain, however, that this species exhibits a physiological rhythm, which is marked by the periodic reversal of its phototropic sign, and by the agglutinative capacity which characterizes the photophobic state.

This physiological rhythm, when observed *in vitro*, is not directly determined by the periodicity of the tide, although it appears to be synchronized to it. In combination with the direct action of light, it controls the rhythmic behavior of the diatoms under the artificial laboratory conditions, and disappears after a few days.

All these facts are in complete agreement with the results of known experiments on the tidal rhythm of *Euglena limosa*, of *Chromulina psammobia*, and of a ciliate associated with symbiotic Chlorella, namely, *Strombidium oculatum*. In the last case (Fauré-Fremiet, 1948), the living conditions are quite different from those of the sand-dwelling species. The above observations lead to the conclusion that the explanation previously suggested for the behavior of the three forms cited above will also apply in the case of *Hantzschia amphioxys*.

Numerous observations and experiments have been made on the rhythmic behavior of simple physiological events. With respect to those which are found in the Protista, as are those described above, it has been presupposed first, that the physiological rhythm is endogenous, and second, that the periodicity which appears to be exactly defined at the population level, is not necessarily so defined in the individual. In addition, it has been assumed that the approximate period for the complete cycle slightly exceeds the 24 hours of a day.

If this is true in the present instance, each rising tide will cause every individual which, at that moment, is not sheltered and agglutinated in the deep layers, to come out of the sand. In other words, a selective elimination takes place at each flood tide, and hence, by elimination of all the "non-conformist" individuals, the syn-

chronism of the endogenous physiological rhythm with the tidal periodicity is maintained.

When this selective elimination is suppressed, as under continued laboratory conditions, the individual variations in periodicity are asserted, and the resulting progressive overlapping of the endogenous periods leads to the disappearance of the collective rhythm.

The statistical maintenance under natural conditions of a synchronism of physiological rhythm and tide is responsible for the apparent adaptation which assures the permanence of a population of small and moving individuals, in a biotope periodically washed by the sea.

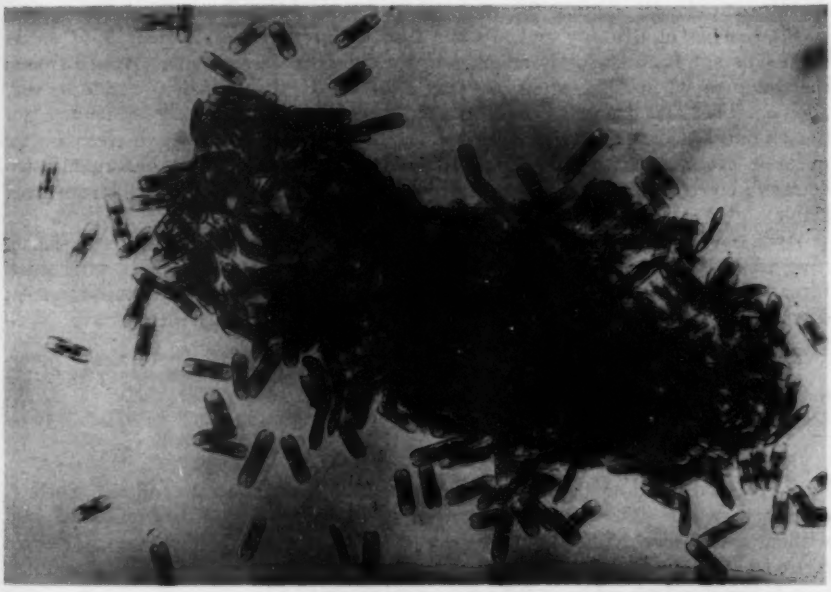

FIGURE 1. A clump of *Hantzschia amphioxys* at the photophobic state, agglutinated on a glass slide under a sand grain layer.

As has been stated above, individuals of *Hantzschia amphioxys* agglutinate to form small clusters in the sub-surface sand when in the photophobic phase. When the same phenomenon is observed in sand on the bottom of a small glass dish or on a microscope slide placed on the bottom of a Petri dish, it may be demonstrated that these clusters adhere to the glass and to the grains of sand. The masses maintain their form and prove to be elastic when they are teased with needles. When fixed while adhering to a slide, it is possible to stain and wash the preparation without freeing the clusters (Fig. 1).

It would appear, therefore, that in the photophobic state, the diatoms are surrounded by a jelly, coagulated by fixative reagents, and possibly a mucoid, which

may be considered as a secretion product. These facts are very suggestive in the light of the common statement that slow coagulation of organic substances plays a role in the flocculation and stabilization of marine sediments.

Many interstitial organisms, such as nematodes, copepods, ciliates, and even the agglutinated diatoms themselves, however, continue to move in the sand. The motion is often sufficiently extensive to result in slight displacements of sand grains which are bound together by diffused organic products. Thus, in spite of a relative fragility, the superficial layers of the sand comprise a relatively stable and coarse framework within which there dwells a permanent interstitial microscopic fauna and flora. This is a fact of some ecological interest.

Marine littoral sands represent peculiar and interesting biotopes with respect to the interstitial microfauna. The comparative study of the sand-inhabiting ciliates of the beaches of Brittany and of Cape Cod (Fauré-Fremiet, 1950a, 1951) shows that some species are closely adapted to the interstitial conditions. When free in the water, or when adjacent surfaces are clean solids, the animals prove to be very fragile. The sand grains of the beach must represent a very complex biotope, for the organic substances which cover them appear to have a protective effect on the unstable cytoplasmic surface of the ciliates. At the same time, the organic substances may cohere to render the "structure" of the superficial sand layers more stable.

It is obvious that the secretory and excretory products of the many sand-inhabiting annelids and molluscs play an important part in the development of sand structure. The above observations show that the same is true for many much smaller organisms; among these are the Protophyta and most certainly the bacteria.

I wish to express my thanks to Dr. L. Hoadley for his kind revision of my manuscript.

Summary

1. A sand-dwelling population of the diatom *Hantzschia amphioxys* Ehrb. is described, which exhibits a tidal rhythm that persists for some days when held without tide under artificial conditions in the laboratory. This rhythm is characterized by a periodic inversion of the phototropistic response, and by an agglutination of the members when in the photophobic state.

2. A comparison of this behavior with that of *Euglena limosa, Chromulina psammobia* and *Strombidium oculatum* shows close analogies and suggests that the same explanation may account for the synchronization of physiological rhythm and tide in all cases. It seems to be that the rising tide acts as a selective factor.

3. Organic substances produced by the diatoms contribute to the structural stabilization of the superficial sandy layer, and the presence of this organic material appears to be an ecological condition essential to the interstitial microfauna.

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THE DESOXYRIBONUCLEIC ACID CONTENT OF THE NUCLEUS AS A CYTOTAXONOMIC CHARACTER IN MANTIDS (ORTHOPTERA: MANTOIDEA)

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The hypothesis that the nuclear content of desoxyribonucleic acid is a constant character of the species is supported by rapidly accumulating evidence (Boivin, Vendrely and Vendrely, 1948; Vendrely and Vendrely, 1948, 1949, 1950; Mirsky and Ris, 1949; Ris and Mirsky, 1949; Swift, 1950a, 1950b; and others). It moreover appears probable that the higher taxonomic categories will show a characteristic order of magnitude with respect to this value; and polyploid species already tested show the expected stepwise relation. The possibility is thus opened that the amount of DNA per nucleus may prove a useful cytotaxonomic tool in evaluating evolutionary relationships among species whose karyotypes are not analyzable by the methods of comparative cytology. The following report records a preliminary exploration of this possibility for certain problems of karyotype relationship among mantids.

Cytological Background

The first group investigated comprises three species of the taxonomically difficult genus *Liturgousa* of the subfamily Liturgousinae. Closely similar in phenotype and habit, and occupying common or adjacent ranges, they differ widely in chromosome complements (Hughes-Schrader, 1950). *L. maya* has 16 autosomes, while *L. cursor* precisely doubles this number with 32, and *L. actuosa* [1] is intermediate with 22. While the X chromosome appears identical in all three, the autosomes are not morphologically homologous, but show in general an inverse relation of size to number (Figs. 1, 2 and 3). No direct Robertsonian relation (the evolutionary equivalence of one mediokinetic to two acrokinetic chromosomes) is demonstrable between *L. maya* and *L. actuosa*, nor between *L. maya* and *L. cursor*. It is possible, however, that *L. cursor* and *L. actuosa* stand in this relation to each other, if the 10 pairs of apparently rod-shaped autosomes of the former correspond to 5 pairs of V-shaped elements in the latter. No final conclusion is justified since the position of the kinetochore in the short chromosomes of *L. cursor* cannot be established positively. On comparative inspection the chromosomes of the three species appear approximately equal in total mass, but measurement at spermatogonial metaphase shows the total length of the chromosomes to vary inversely with chromosome number (Table I). The total cytological evidence thus fails to make clear the evolutionary relationship of these species. While precluding polyploidy, it suggests

[1] The erroneous name *L. arcuosa* was used for this species by Hughes-Schrader (1950). Its correct designation and description have since then become available in Rehn, 1951, *Trans. Ent. Soc.* **76**: 363–383.

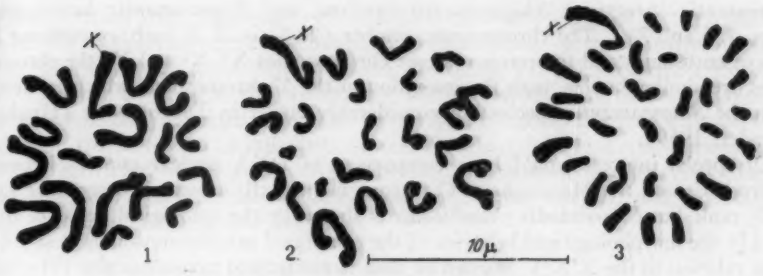

FIGURE 1. Spermatogonial metaphase—*Liturgousa maya*.
FIGURE 2. Same—*Liturgousa actuosa*.
FIGURE 3. Same—*Liturgousa cursor*.

(All drawings made with camera lucida, at table level; Zeiss apochrom. obj. 90, comp. oc. 20; magnification as reproduced is uniform in all and indicated by scale on each. Fixation Sanfelice; stain Feulgen.)

that changes in total amount of chromosome material, as well as changes in its distribution and in number of kinetochores, have been associated with this divergence. Measurement of metaphase chromosomes in any species can, however, at best give but an approximate index of the amount of chromosome material, for differences in intensity of dye and tightness of coiling cannot be adequately appraised. It was therefore hoped that the photometric determination of the relative amount of DNA in the interphase nucleus, by providing a more accurate index of the substance measured in Feulgen-dyed metaphase chromosomes, might elucidate the interrelationship of the *Liturgousa* species.

With the dual purpose, first, of providing a standard of comparison for possible interspecific differences in the nuclear DNA content among the *Liturgousa* species, and second, of ascertaining whether or not cytotaxonomically useful constants of nuclear DNA content characterize different subfamilies, measurements were also made on a second group of mantids from the very distantly related subfamily Manteinae. These were chosen on the basis of close similarity in karyotype, and include

TABLE I

Average total length, in arbitrary units, of metaphase chromosomes

Species	Chromosomes $2n\,♂$	Spermatogonia	Number measured	Meiosis II	Number measured
Liturgousa maya	16+X	59.9	3		
Liturgousa cursor	32+X	42.6	3		
Liturgousa actuosa	22+X	51.5	3		
Choeradodis rhombicollis	28+X^1X^2Y	56.3	3		
Stagmomantis carolina	24+X^1X^2Y	62.5	4	53.6	4
Stagmomantis heterogamia	24+X^1X^2Y	68.2*		58.6	6
Tauromantis championi	24+X^1X^2Y	66.0	5		
Pseudomiopteryx infuscata	16+X	26.9*		23.1	8

* Calculated from ratio of total length of meiotic to gonial chromosomes in *Stagmomantis carolina*.

Tauromantis championi, *Stagmomantis carolina*, and *Stagmomantis heterogamia* (Figs. 4, 5 and 7). The chromosome number ($2n♂$) is 27 in each, comprising 12 pairs of autosomes and the compound sex chromosomes X^1, X^2 and Y; the chromosomes are similar in size, with the exception of the Y chromosome, which is identical in the *Stagmomantis* species but considerably larger in *Tauromantis* (Hughes-Schrader, 1950).

Of special interest would be a comparison of DNA nuclear content between *Choeradodis* and the Manteinae. The genus is currently elevated to separate subfamily rank, but *Choeradodis rhombicollis* is shown by the cytological evidence provided by the morphology and behavior of the compound sex chromosomes to stand in closer relation to the X^1X^2Y Manteinae than is recognized taxonomically (Hughes-Schrader, 1950). The autosomes of *Choeradodis*, while numbering two more pairs than are found in the Manteinae, are individually considerably smaller and their total length appears approximately the same as in the latter. Unfortunately, the only

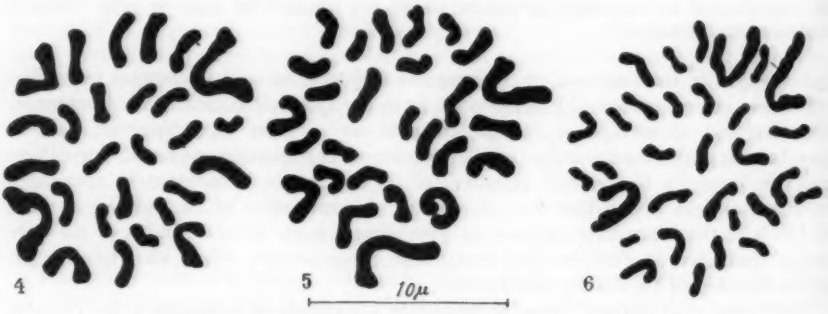

FIGURE 4. Spermatogonial metaphase—*Stagmomantis carolina*.
FIGURE 5. Same—*Tauromantis championi*.
FIGURE 6. Same—*Choeradodis rhombicollis*.

material of *Choeradodis* available for photometry gives evidence of considerable cytological abnormality; the results obtained from it can be regarded as suggestive only.

Finally, measurements were made on nuclei of *Pseudomiopteryx infuscata* of the subfamily Pseudomiopteriginae. Here, while the chromosome number ($2n♂ = 17$) is the same as in *Liturgousa maya*, the total mass of the chromosomes appears to be far less. When compared with the manteine species *Stagmomantis heterogamia*, the total length of the chromosomes of the *Pseudomiopteryx* complement is seen to be less than half that in the first named species (Table I and Figs. 7 and 8). The nuclear DNA content of such a karyotype is of special interest since the distribution of chromosome numbers among all cytologically known mantids shows two peaks—a dominant one at 27 with outlying species ranging upwards to 39, and a secondary peak embracing the 15 to 19 chromosome range. Furthermore, in many of the high-number karyotypes the X chromosome (or its derivative arms in X^1 and X^2) is of extreme length relative to the autosomes and in comparison with the X of certain low-number karyotypes such as that of *Pseudomiopteryx*. Thus, the

possibility exists that polyploidy—its establishment made possible by the stabilization of the sex chromosome mechanism through fusion of the X chromosomes, along lines similar to those first postulated by Bauer (1947) for certain Dermaptera—has been involved in the evolution of the Mantoidea. Of course no implication of any recently established diploid to tetraploid relation between *Pseudomiopteryx* and *Stagmomantis* is intended; taxonomic and cytologic considerations alike preclude it. They were chosen, from the limited material available, as roughly representative in general features of chromosomal mass, number, and morphology of the postulated ancestral karyotypes.

Material and Methods

The material used comprises testes from males of the 8 species enumerated above; collection and field notes have been recorded previously (Hughes-Schrader,

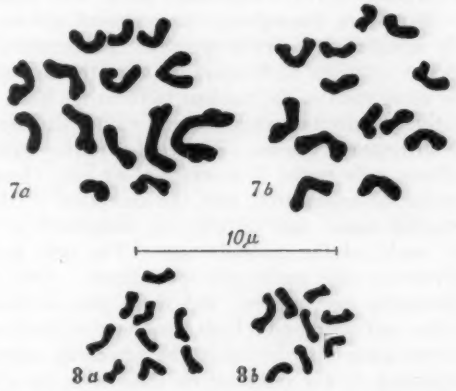

Figure 7. Second meiotic metaphase—*Stagmomantis heterogamia*. a. Secondary spermatocyte with X^1 and X^2; b. Secondary spermatocyte with Y.

Figure 8. Second meiotic metaphase—*Pseudomiopteryx infuscata*. a. Secondary spermatocyte with X; b. Secondary spermatocyte with no X.

1950). For photometric measurements two individuals of each of the three species of *Liturgousa*, and one of each of the other 5 species, were used. The material was fixed in Sanfelice, washed in water overnight, embedded in paraffin and sectioned at 8 or 10 μ. Sections from each of the *Liturgousa* species were mounted together on consecutive slides, as were those of the Manteinae-*Choeradodis* group. In addition the slides of the latter series carried sections from one testis of the first group to provide a standard for comparison of data from different slides. The limited material available restricted direct comparison of *Pseudomiopteryx* to *Stagmomantis heterogamia* and *Choeradodis* of the second group.

Staining was by the Feulgen method; alternate slides in each series constituted unhydrolyzed controls. The photometric measurements were made by the Pollister and Ris (1947) method and with the apparatus described by Swift (1950a). DNA in the nucleus was measured as the extinction of the Feulgen dye at the 546 mμ line, isolated by a Farrand Interference Filter No. 2756 from an AH4 mercury

vapor lamp. The relative amount of DNA per nucleus was calculated in arbitrary units of Feulgen dye, according to the formulae given by Swift (1950a): the measured extinction of a cylinder 3.8 μ in diameter through an uncut nucleus is multiplied by the squared radius of the cylinder and divided by the percentage of the total nuclear volume represented by the cylinder. The nuclei measured deviated only slightly if at all from the spherical; major and minor axes were measured and the mean considered as the diameter. Feulgen preparations following Sanfelice fixation of mantid testes often show, in control and test slide alike, a diffuse stain in the cytoplasm. Since the intensity of this cytoplasmic dye varied significantly among some of the species tested, the extinction of each nucleus measured was corrected by a factor relating the amount of overlying cytoplasm to the cytoplasmic extinction.

Spermatid nuclei were chosen for measurement since they may be expected to be relatively free of variation in DNA associated either with mitosis or with special metabolic function. Moreover, throughout the series of species tested, spermatid nuclei proved highly comparable morphologically. Fortunately also, in all these species, the chromosomes continue to de-spiralize after the second meiotic telophase and, just prior to the elongation of the nucleus to form the head of the sperm, they reach a state of relatively extreme extension and rather diffuse staining which is favorable for photometric measurements. To avoid variation stemming from differential penetration effects, only nuclei from cysts at or very close to the surface of the testis were utilized. All testes used, with the exception of the *Choeradodis* material, are from nymphal males and provide an abundance of uniform, normal-appearing spermatid nuclei of the desired stage. The only available material of *Choeradodis* comes from an adult male with spent testes. Only a few cysts of the required stage of spermatid are present; and these give evidence—through wide variation in nuclear size and stain and a high frequency of giant and micro nuclei—of abnormality in chromosome distribution in the preceding mitosis.

To facilitate correlation of the photometric data with the cytological findings, estimates were made of the total length of the chromosomes at spermatogonial or second meiotic metaphase in Feulgen preparations. It was found that a reasonable agreement in values was obtained only from the relatively few plates in which all chromosomes were extended fairly flatly in one plane; the figures on total length in Table I are the mean values from measurements of from three to eight such metaphase plates. The maximum deviation from the average did not exceed 8 per cent, except in *Tauromantis* and *Choeradodis* plates, where it reached 14 per cent. Camera lucida drawings, at 1800 ×, were enlarged four fold by pantograph and the length of each chromosome recorded with a map measure. It proved impossible to gauge the diameter of the chromosome satisfactorily by this method; and since differences in chromosome diameter are distinguishable among these species, the total chromosome length can give only an approximation of total mass.

Results

The photometric measurements of the relative amount of DNA in spermatid nuclei disclose a range of values characteristic for each of the species tested. The results are summarized in Table II, in which each major vertical column contains data from a single slide. For brevity in presentation the results are recorded as the

mean values of the different samples of nuclei measured. The use of the mean DNA per nucleus value as a species constant appears justified for purposes of preliminary interspecific comparison; to what extent the spread of values about the mean reflects real variation among the nuclei, and to what extent errors in method and measurement, is unknown. The pattern of scatter of the values obtained does show the effect of the segregation of the sex chromosomes, especially in the spermatids of the manteine species where two relatively large X chromosomes have segregated from a small Y. When DNA content is plotted against number of nuclei, some indication of a bimodal distribution is apparent in each sample. It is also of interest to note, relative to the variation within the species range, that no nucleus measured contains DNA in an amount equal to or closely approaching twice the mean value, and in only four instances was the maximum value twice that of the minimum obtained.

TABLE II

Average amounts (in arbitrary units of Feulgen dye) of DNA in spermatid nuclei of mantid species. (a,b = 2 individuals tested; N = number of nuclei measured)

Species	Slide 1			Slide 2			Slide 3			Slide 4			Slide 5			Total number of nuclei measured
	DNA	S.E.	N	DNA	S.E.	N	DNA	S.E.	N	DNA	S.E.	N	DNA	S.E.	N	
Liturgousa maya a	1.11	0.041	14	1.04	0.049	16										30
Liturgousa maya b				1.06	0.026	15										15
Liturgousa cursor a	1.01	0.026	10	0.98	0.027	15										25
Liturgousa cursor b										1.04	0.029	20				20
Liturgousa actuosa a	1.51	0.041	15	1.48	0.049	16	1.50	0.059	12	1.43	0.025	20				63
Liturgousa actuosa b										1.65	0.020	20				20
Choeradodis rhombicollis							1.52	0.086	20				1.18	0.027	36	56
Stagmomantis carolina							1.80	0.073	20							20
Stagmomantis heterogamia							1.65	0.060	20				1.59	0.041	26	46
Tauromantis championi							1.73	0.077	17							17
Pseudomiopteryx infuscata													0.74	0.024	20	20

Before turning to a consideration of the results in relation to the cytological problems presented, one other point of general interest should be emphasized. As shown in Table II (slides 2 and 4), the two individuals of *Liturgousa maya* tested are practically identical in respect to the DNA content of spermatid nuclei; the same is true of the two specimens of *L. cursor*. In *L. actuosa*, however, a significant difference is apparent (Table II, slide 4)—the second individual tested shows a value 15 per cent higher than that of the first. In view of this finding, the number of individuals of each species sampled in the present study must be considered as too low to justify final conclusions as to the limits of the specific range in the DNA value of the spermatid.

DISCUSSION

Liturgousinae

In the three species of *Liturgousa* studied, it is clear (Table II, slides 1 to 4) that the amount of DNA per spermatid nucleus does not vary directly with chromosome number and size. The nuclei (haploid) of *L. maya* with 8 autosomes and

those of *L. cursor* with precisely twice as many show no significant difference in DNA content. This is in agreement with the tentative conclusion based on cytological data (Hughes-Schrader, 1950) that a re-distribution of chromosome material and a change of kinetochore number not involving polyploidy has been associated with the evolutionary divergence of these species. In view of the 1:1 ratio in DNA it is, however, surprising to find a ratio of 1.4:1 in the total length of metaphase chromosomes between *L. maya* and *L. cursor* (Table I); moreover, the *L. maya* chromosomes are appreciably thicker than those of *L. cursor*. This discrepancy may be more apparent than real in respect to the actual amount of Feulgen-positive material present, since considerable differences in intensity of stain and in degree of condensation of the metaphase chromosomes are not detectable by the eye.

But by far the most interesting result of the comparison of the *Liturgousa* species, and one not to be anticipated from the cytological evidence, is the wide divergence in DNA values of *L. actuosa* from its two sister species. Closely similar to *L. maya* and *L. cursor* in phenotype, intermediate between them in chromosome number and size and in total chromosome length, *L. actuosa* is characterized by an amount of DNA per spermatid which is half again as large as theirs. No differential polyteny among the chromosomes is discernible at the mitosis preceding spermatid formation; and in chromosome diameter—admittedly difficult to gauge with accuracy—*L. actuosa* appears to be intermediate between *L. maya* and *L. cursor*. That a change of such magnitude in the relative amount of DNA per nucleus may be effected among closely related species without a corresponding visible change in total chromosomal mass is, aside from its interest relative to chromosome structure, prejudicial to the hope that a DNA species constant will prove cytotaxonomically useful. The significance of this finding is of course dependent on the validity of my assumption that the spermatid nuclei of the species concerned are actually as comparable in respect to the timing of DNA synthesis as they appear to be in all cytologically demonstrable characters. If any synthesis, possibly anticipatory of the next mitosis, takes place in the spermatid nucleus, it may well have undergone interspecific variation in time or rate. It is thus desirable that the DNA content of the spermatid be compared to that of somatic nuclei in each of the species involved; unfortunately, this must await the collection of further material.

Manteinae

Uniformity in spermatid DNA characterizes the three species of the subfamily Manteinae—*Tauromantis championi, Stagmomantis carolina,* and *Stagmomantis heterogamia;* the mean values obtained (Table II, slide 3) do not differ significantly, and in range of values the four samples of nuclei measured are practically identical. This is in harmony with the cytological findings, for these species have the same number of chromosomes and are closely similar in chromosome morphology and behavior. In total length of chromosomes as measured at spermatogonial metaphase also, *Tauromantis* and *Stagmomantis carolina* are in good agreement (Table I). No measurable spermatogonial metaphases are available for *Stagmomantis heterogamia,* but at second meiotic metaphase the total length of its chromosomes agrees, within the probable error of measurement, with that of *S. carolina.* Assuming the same ratio in length between meiotic and gonial chromosomes in the

two *Stagmomantis* species, the total chromosome length at spermatogonial metaphase in the three manteines (Table I) shows no greater variation than is obtained in measurements of different plates in one species.

Relationships among X^1 X^2 Y mantids

The species of the subfamily Manteinae characterized by the compound sex chromosomes—X^1X^2Y ♂—$X^1X^1X^2X^2$ ♀—form a closely linked natural group cytologically—their joint possession of this particular sex chromosome mechanism denotes, with a high degree of probability, their descent from a single ancestral species (White, 1941). Species with a sex trivalent strikingly similar in structure and behavior are now known in four other subfamilies, and the cytological evidence indicates a closer relation to the Manteinae than is expressed in their taxonomic placement (Hughes-Schrader, 1950). Of these non-manteine X^1X^2Y species, material of only one—*Choeradodis rhombicollis*—was available for comparison of DNA values with the Manteinae. And as noted above, the few cysts of spermatids remaining in this adult testis give evidence of abnormalities in chromosome distribution in the preceding mitosis, which precludes a determinaton of the DNA constant of the species. The mean value for DNA in the spermatid in the two disparate samples measured, however, falls below that of the Manteinae (Table II, slides 3 and 5); and since not one nucleus in 56 measured exceeded in DNA the maximum value obtained in the Manteinae, we may expect the real value for *Choeradodis* to be somewhat lower than the general manteine level. This is of interest since *Choeradodis* has two more pairs of autosomes than is characteristic for X^1X^2Y Manteinae. The cytological evidence also indicates that the difference in chromosome number implies no increase in chromosome mass, for the total length of the spermatogonial chromosomes is approximately the same (Table I), and their diameter is appreciably less (Figs. 4, 5 and 6), than in the manteine species. As to the major issue, however—the relationship of X^1X^2Y species currently referred to different subfamilies—more normal material of *Choeradodis* than presently available is obviously necessary in order to test the relevancy of photometric data on nuclear DNA.

Polyploidy

Pseudomiopteryx of the subfamily Pseudomiopteriginae was chosen for photometric measurement of nuclear DNA content because, as noted previously, it represents a group of low-number karyotypes among mantids which apparently contain not more than half the Feulgen-positive chromosome material present in many high-number karyotypes such as the three species of the subfamily Manteinae here tested. This in turn suggests that polyploidy may have been involved in the early differentiation of high and low chromosome numbers in ancestral mantid stocks. The photometric data (Table II, slide 5) do indeed support this suggestion: the relative amounts of DNA per spermatid in *Pseudomiopteryx* as compared with *Stagmomantis heterogamia* closely approach a 1:2 ratio (ratio = 0.47). A greater disparity is apparent in total length of chromosomes at the last preceding mitosis (Table I); the ratio here is 0.39. Since, however, the arms of second meiotic metaphase chromosomes of both species tend to curl at the tips and the resulting error in measurement is proportionately greater in the shorter chromosomes of *Pseudomiopteryx*, this finding need not be considered wholly out of line with the photo-

metric data. On the other hand, the difference in diameter between the chromosomes of *Stagmomantis* and *Pseudomiopteryx*, as shown in Figures 7 and 8, gives the impression of a greater disparity in total mass than is indicated by the ratio in total length.

I am convinced that polyploidy has played a greater role in the evolution of animal karyotypes than was earlier so generally assumed. Nevertheless, the hypothesis that polyploidy was involved in the evolution of the Mantoidea must be advanced with reservations. In the first place, the chromosome numbers now known among mantids embrace nearly all intermediate values between the low and high peaks. Secondly, the photometric data here reported do not exclude the possibility of a differential polyteny in the spermatids of the different species considered. And finally, a change of great magnitude in the DNA content of spermatid nuclei has been established within a group of three congeneric species where the total evidence almost certainly excludes polyploidy. *Liturgousa actuosa* has approximately one and one half times the amount of DNA per spermatid that is found in *L. maya* and *L. cursor;* two changes of like nature and magnitude would result in a 2:1 ratio with no implication of polyploidy. The possibility of a polyploid relation between low and high number karyotypes suggested by the spermatid DNA ratio between *Pseudomiopteryx* and *Stagmomantis heterogamia* is, however, open to more critical tests which it is hoped to prosecute.

DNA values as subfamily characters

Aside from the detection of polyploid relationships, the utility of DNA determinations in cytotaxonomic analysis will depend largely on whether or not the lower categories such as subfamilies possess characteristic and distinguishable relative amounts. The present data, though stemming from far too few species to answer this question, are suggestive in relation to it. The subfamilies Pseudomiopteriginae and Manteinae, as here sampled, show a wide separation between the mean values of spermatid DNA, and there is no overlapping in the range of values obtained from the two groups; should these values prove characteristic for the subfamilies, they will constitute valid cytotaxonomic criteria. In the subfamily Liturgousinae, exclusive of *Liturgousa actuosa*, the mean amount of spermatid DNA differs significantly from that of the Manteinae on one side and from the Pseudomiopteriginae on the other, although in range of values there is wide overlapping. But *Liturgousa actuosa*, in which the mean amount of DNA per spermatid does not differ significantly from that of *Stagmomantis heterogamia*, bridges the gap between Liturgousinae and Manteinae. Since taxonomical and cytological evidence utterly preclude consideration of *Liturgousa actuosa* as a bridging form between these very distantly related subfamilies, this case alone sharply restricts the potential usefulness of the relative amount of DNA in the spermatid nucleus as a cytotaxonomic criterion.

Summary

1. The relative amount of DNA per spermatid nucleus has been determined by photometric microscopic measurement in eight species of mantids, and the results are discussed in relation to their cytology and the cytotaxonomic problems they present. The mantids studied are the following: Subfamily Liturgousinae—*Litur-*

gousa maya S. & Z., *L. actuosa* Rehn, *L. cursor* Rehn; Subfamily Choeradodinae —*Choeradodis rhombicollis* Latr.; Subfamily Manteinae—*Tauromantis championi* S. & Z., *Stagmomantis carolina* Johann, *S. heterogamia* S. & Z.; and Subfamily Pseudomiopteriginae—*Pseudomiopteryx infuscata* S. & Z.

2. Of the three closely similar species of *Liturgousa* tested, *L. maya* with 16 autosomes and *L. cursor* with 32 have the same content of DNA in the spermatid nucleus, confirming the cytological evidence that no polyploid relation exists between them. The greater diameter and greater total length of metaphase chromosomes in *L. maya* as compared with *L. cursor* remain unexplained.

3. *Liturgousa actuosa* has approximately one and one half times the amount of spermatid DNA as *L. maya* and *L. cursor*, in marked disagreement with the cytological evidence for an intermediate position.

4. *Tauromantis championi* and the two species of *Stagmomantis*, of the Manteinae, are uniform in DNA spermatid values, as in karyotypes. For *Choeradodis* no reliable species constant in nuclear DNA could be determined, due to abnormalities in the material, but a value not exceeding that of the Manteinae is indicated; this is in harmony with the cytological evidence.

5. The ratio of DNA per spermatid nucleus in *Pseudomiopteryx infuscata* to that in *Stagmomantis heterogamia* is 0.47; the ratio in total length of chromosomes at the preceding metaphase is 0.39. The implications of these findings for the hypothesis that polyploidy has been involved in the evolution of high and low number karyotypes among mantids are considered.

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THE UPTAKE OF RADIOACTIVE IODINE BY ASCOPHYLLUM

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During the course of experiments designed to determine the relation between respiration and iodine uptake in brown algae (to be published elsewhere), it was observed that the uptake of radioactive iodine followed over a period of 15–20 hours gave a definite pattern of behavior. This has led us to interpret the uptake of iodine from sea water as involving a constant exchange process between iodine within the algae and iodine in the surrounding medium.

The interpretation of the uptake of substances by plants as a continuous exchange phenomenon by means of tracer experiments heretofore has been made by studying the movement of ions between roots and soil colloids (Jenny and Overstreet, 1938; Jenny, Overstreet and Ayers, 1939); also by movement of ions between roots and culture solution (Overstreet and Broyer, 1940; Broyer and Overstreet, 1940; Overstreet and Jacobsen, 1946; Jacobsen and Overstreet, 1947; Broyer, 1950; Russell and Martin, 1950).

The observations reported here are unique in that they involve the uptake process of a trace, rather than a major element, and they shed light on the nature of anion, rather than cation, uptake. They are also basic to the correct interpretation of further uptake experiments with the isotope.

Segments of the brown alga, *Ascophyllum nodosum* (Linn.) LeJolis, were measured for their uptake of iodine^{131} after various intervals of exposure to the isotope. Segments cut one cm. in length and washed 12–16 hours in running sea water were placed in 25 ml. volume Erlenmeyer flasks containing 3 ml. of Van't Hoff artificial sea water, to which had been added potassium iodide to give a concentration of 0.05 p.p.m. iodide (the approximate concentration in natural sea water). The isotope, iodine^{131} with no carrier added, supplied in $NaHSO_3$, was added to the solution in amounts giving approximately 14 μc. per experimental flask.

The absorbed iodine^{131} was determined after exposure to this environment for a given time interval by obtaining counts of the radioactivity present in the alga segments. Before each count was taken the segments were removed and washed by six successive rinses with a non-radioactive solution, which otherwise was similar to that in the flask. Each rinse contained 15–20 ml. of the solution. It was not possible to estimate how much of the radioactive iodine found in the rinsings came from the surface and how much from that actually absorbed by the alga. That the latter was small or at least constant was indicated by the fact that the sixth rinse showed no activity, independent of the concentration of radioactive iodine. The excess wash solution was blotted from the segments with a paper towel. The segments were then transferred to a counting pan, 10 to a pan, in such a manner that

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[2] This document is based on work performed under Contract AT-30-1-Gen-70 for the Atomic Energy Commission.

constant geometry was maintained for all sets of samples. After counting, they were returned to the radioactive solutions for the next time interval.

Counting rates for the solution and that remaining after the segments had been removed were determined by the use of 2 ml. aliquots in small sample pans. This constituted a thick source for the iodine betas. These counting rates made it possible to compare the shape of the curve for iodine^{131} remaining in the solution with that for iodine taken up by the alga segments.

All counting rates were determined by taking at least 2×10^3 total counts, so that the statistical error is below two per cent. The counting rates thus determined were then corrected for background and decay of the iodine^{131}.

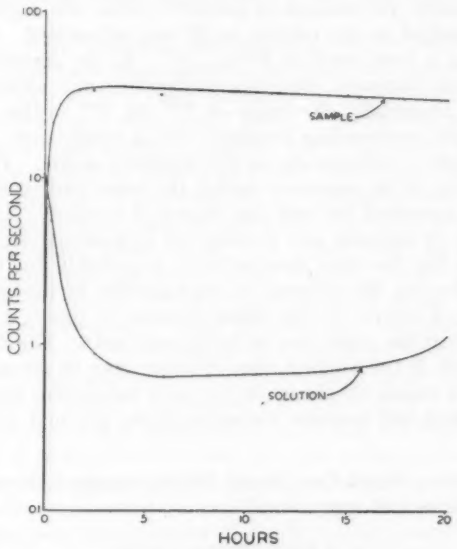

FIGURE 1. Uptake of iodine^{131} by alga segments. This curve represents a typical experiment. Comparison of counting rates of the sample and solution is not possible because of their differences in counting geometry.

These experiments were also performed using natural sea water. This was done for the purpose of determining whether a possible difference in iodine concentration (between artificial and natural) in the sea water would affect the shape of the curves. The character of the curves did not change.

A study of Figure 1 gives the following information about the uptake of iodine^{131} by the alga segments:

1. The concentration of iodine within the segments remains the same throughout the experimental period. This is shown by the fact that a steady state of iodine^{131} content is reached eventually, and no further increase in radioactive iodine uptake is observed.

2. It is characterized by an exchange phenomenon between iodine within the

segments and iodine in the surrounding medium. This interpretation is based on the assumption that since the alga had been living for a long period of time in an iodine-present medium, natural sea water, additional uptake of iodine could occur for either of two reasons: 1) iodine is being used at a constant rate for some life process, or 2) iodine is constantly moving in and out in a dynamic exchange process. The attaining of an equilibrium, expressed in the plateau of the curve, leads us to conclude that iodine is being taken up for the latter reason.

The large slope which is obtained initially shows that this exchange is carried on at a rapid rate. The decrease in slope and the leveling off are due to the fact that initially the segments do not contain any radioactive iodine, but as the process goes on the concentration of I^{131} to I^{128} becomes equal inside the segments and in the solution. Initially, the amount of iodine^{131} within the segments is nil and the iodine being exchanged to the outside is all non-radioactive. It is replaced by a solution containing a fixed ratio of I^{131} to I^{128}. As the concentration of iodine^{131} within the segments increases, both non-radioactive and iodine^{131} are exchanged to the solution. Eventually the ratio of I^{131} to I^{128} within the segments approaches that of the surrounding medium (*i.e.*, a steady state exists) and no increase in the amount of radioactivity in the segments occurs. The slight but steady drop in radioactivity of the segments during the latter part of the experimental period has not been accounted for with any degree of certainty. In many cases iodine has been found to be unstable and to come off in gaseous form. Preliminary experiments indicate that the drop observed here is probably due to a loss of I^{131} as a gas. Curve B, showing the decrease in radioactivity of the solution corroborates the interpretation of Curve A: the initial decrease is rapid; a steady state in the solution is reached at the same time as in the segments. The rise in concentration of radioactive iodine in the solution after 16 hours may be accounted for by evaporation. Although it causes no change in the total radioactive iodine content, evaporation of the solution will increase the radioactivity per unit volume, which Curve B measures.

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STUDIES ON THE ISOLATED ISLET TISSUE OF FISH. I. THE CYTOCHROME OXIDASE AND SUCCINIC DEHYDROGENASE CONTENTS OF NORMAL TOADFISH (OPSANUS TAU)

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A determination of the metabolic characteristics of the insulin-producing beta cells of the pancreas has been undertaken in the hope that these studies may offer an explanation as to why the beta cells are selectively killed by toxic agents such as alloxan (Dunn, Sheehan and McLetchie, 1943). Several hypotheses have been advanced to explain this selectivity (Lazarow, 1949; Lazarow, Liambeis and Jan Tausch, 1950) but these must be verified by direct chemical and enzymatic analyses of the insulin-producing cells. Unfortunately, it is difficult to study the metabolism of the beta cells in mammalian species because the islet tissue is inextricably intermingled with the pancreatic acinar tissue. In teleost fish, however, the insulin-secreting cells are segregated into one or more discrete bodies called the principal islet (located in the mesentery), whereas the pancreatic acinar tissue is diffusely dispersed throughout the mesentery, along the bile ducts, and within the liver (Diamare, 1899; Rennie, 1905). The isolation of insulin from the principal islet tissue of teleost fish and its absence from the diffuse acinar tissue suggested that islet tissue was the source of insulin in mammals (McLeod, 1922). Although the fish islet tissue contains several cell types (Jackson, 1922), it may be presumed by analogy to mammalian studies that it is the beta cells which produce insulin. Since alloxan diabetes and beta cell destruction has been produced in fish (Lazarow and Berman, 1947, and also unpublished observations) it may be possible to deduce information about the metabolic specialization of the beta cells themselves by comparing normal islet tissue with islets obtained from alloxan diabetic fish (*i.e.,* islets with decreased numbers of insulin producing cells).

Unfortunately the islet tissue of a 200 gm. toadfish weighs between 1 and 2 mg. and therefore it has been necessary to undertake this study by ultramicro analytical techniques. We have begun a systematic investigation of the enzymes and metabolic activities of normal fish islet tissue; the cytochrome oxidase and succinic dehydrogenase contents are herein reported. It is hoped that these studies may help to explain the mechanism by which alloxan produces selective necrosis of the beta cells, and that perhaps they may contribute to our understanding of the etiology of human diabetes. They may also furnish information useful for the cultivation of islet tissue *in vitro,* a problem which is becoming increasingly important in view of the approaching shortage of insulin.

Methods and Materials

Mature toadfish weighing between 200 and 600 gm. were kept in a running sea water tank for one to seven days prior to use. The animals were decapitated and

samples of the various organs were removed and placed in a beaker at 0° C. The principal islet was usually found near the spleen between the layers of the mesentery. The capsule of the islet was dissected and removed. The entire islet and pieces of the other organs weighing between two and 20 mg. were placed between two layers of stretched Parafilm and weighed on a Roller-Smith Micro Torsion Balance to the nearest 1/100th of a milligram.[1]

The weighed tissue was transferred to a conical-tipped micro homogenizer (Lazarow and Portis, 1951) previously cooled to 0° C. The Parafilm was reweighed, and five volumes of fluid were added to the homogenizer with a syringe buret (Lazarow, 1950). The tissues were homogenized by hand, and after appropriate dilution the enzyme contents were determined. The order in which the different tissues were assayed was varied systematically from day to day.

Succinic dehydrogenase was determined by measuring the rate of reduction of cytochrome *c* under standardized conditions in the presence of sufficient cyanide to inhibit any cytochrome oxidase present (Cooperstein, Lazarow and Kurfess, 1950). The tissue was homogenized in 5 volumes of sodium succinate (0.5 *M* in 0.2 *M* PO_4 pH 7.4) and the homogenate was diluted with the same solution to give a final tissue dilution which ranged from 1 : 20 for islet to 1 : 300 for heart. Two determinations were carried out on each sample by mixing 10 or 20 μl. of the tissue homogenate, 10 μl. of a cyanide solution and 280 μl. of a cytochrome *c*-salt solution. The extinction was followed for three minutes at 550 mμ. The enzyme activity of a standard tissue dilution was calculated as previously described (Cooperstein, Lazarow and Kurfess, 1950) and is proportional to the Δlog [ferri-cytochrome *c*]/minute. It represents the change observed in the logarithm of the concentration of oxidized cytochrome *c* per minute if one mg. of tissue is added to 100 μl. of the cytochrome *c*-cyanide solution.[2]

Cytochrome oxidase was measured by determining the rate of oxidation of reduced cytochrome *c* (Cooperstein and Lazarow, 1951). The tissues were homogenized in 5 volumes of *M*/30 phosphate buffer, pH 7.4, and diluted with the same buffer to give a final tissue dilution ranging between 1 : 20 and 1 : 150. Two or 4 μl. of the diluted tissue preparation were added to 300 μl. of the reduced cytochrome *c* solution. The enzyme activity for a standard tissue dilution was calculated and it is proportional to the Δlog [ferro-cytochrome *c*]/minute. It represents the change in the logarithm of the concentration of reduced cytochrome *c*/minute when one mg. of tissue is suspended in 100 μl. of cytochrome *c* solution.[3]

[1] Tissues included between two layers of Parafilm do not dry out (weights remain unchanged $\pm$ .02 mg. over a two-hour period).

[2] Example: When 10 μl. of a 1 : 20 islet tissue were added to 290 μl. of a cytochrome *c*-cyanide solution and the logarithms of the extinctions (corrected for the blank) at 550 mμ were plotted against time, the Δ log [cytochrome c^{+++}]/minute was equal to .088.

$$\text{The standard enzyme activity} = \frac{\Delta\log \text{ cyt. } c \times \text{dilution} \times 300}{\text{volume tissue suspension used} \times 100}$$

$$= \frac{.088 \times 20 \times 300}{10 \qquad 100} = .528.$$

[3] Calculated as in footnote 2.

Results

The average succinic dehydrogenase activity of toadfish islet and of other selected fish tissues are arranged in the order of their activity and are shown in Table I. The final tissue dilutions under the conditions of the assay are recorded, as well as the average value of 10 determinations carried out on 10 separate fish. The standard deviations [4] and the p values are also recorded. The p value recorded represents the statistical significance of each average value as compared to the tissue of next lower activity.

It was found that islet tissue is included in the group of five tissues (testis, ovary, gill and muscle) that have the lowest succinic dehydrogenase activity. The

Table I

Succinic dehydrogenase content of toadfish tissues

Tissue	No. of fish studied	Actual tissue dilution used during assay	Average standard activity $\Delta \log$ [cyto. c] /min. for a 1:100 tissue dilution	Standard deviation σ	P value compared to next lower value
Heart	10	1:9000	4.12	±0.78	<.0001
Liver	10	1:3000	1.24	±0.22	<.0001
Kidney	10	1:1500	.72	±0.17	.102
Brain	10	1:1200	.62	±0.11	<.0001
Islet	10	1:600	.26	±0.05	.88
Testis	10	1:600	.25	±0.03	.44
Gill	10	1:600	.24	±0.06	.75
Muscle	10	1:600	.23	±0.05	.60
Ovary	10	1:600	.22	±0.04	

individual values in this group are not significantly different from one another. Kidney and brain are each about 2½ times as active as islet. Liver and heart are, respectively, about 5 and 20 times as active as is islet tissue.[5]

The average cytochrome oxidase activities of tissues obtained from 10 different fish are recorded in Table II. Gill and muscle have a somewhat lower cytochrome oxidase content than does islet tissue, whereas brain, ovary and testis have a somewhat higher activity than islet (26 per cent, 23 per cent, and 44 per cent, respectively). Kidney and liver have approximately three times and heart approximately six times as much cytochrome oxidase as does islet.[5]

Cytochrome oxidase is the only known enzyme (or enzyme complex) in animal tissues involved in the biological oxidation of cytochrome *c*. By contrast, a number of dehydrogenases, including succinic dehydrogenase, are involved in the biological reduction of cytochrome *c*. It was of interest, therefore, to calculate the ratio of the enzymes which both oxidize and reduce cytochrome *c*. The succinic dehydrogenase: cytochrome oxidase ratios are plotted in Figure 1. Although the value of this ratio is very slightly greater for islet tissue than for ovary or testis,

[4] $\sqrt{\frac{\Sigma(\text{difference})^2}{N}}$.

[5] The relative enzyme activities for different tissues are unchanged when the results are converted to a dry weight basis.

TABLE II

Cytochrome oxidase content of toadfish tissues

Tissue	No. of fish	Final tissue dilution used in assay	Average standard activity Δlog [cyto. *c*]/min. for 1:100 tissue dilution	Standard error σ	P value compared to next lower tissue
Heart	10	1:22,500	10.5	1.97	<.0001
Kidney	10	1:15,000	6.06	0.84	.76
Liver	10	1:15,000	5.96	0.72	<.0001
Testis	10	1:6,000	2.64	0.25	.028
Brain	10	1:6,000	2.31	0.39	.76
Ovary	10	1:6,000	2.27	0.25	<.0001
Islet	10	1:4,500	1.83	0.21	<.0001
Gill	10	1:3,000	1.40	0.21	.0010
Muscle	10	1:3,000	1.12	0.15	

the ratio of succinic dehydrogenase (which reduces cytochrome *c*) to cytochrome oxidase (which oxidizes cytochrome *c*) is less in islet tissue than in any of the other tissues studied with the exception of kidney. Even though the succinic dehydrogenase: cytochrome oxidase ratio in toadfish kidney is somewhat lower than in islet tissue, the absolute amount of succinic dehydrogenase present was almost three times that of the islet tissue.

In order to determine whether the enzyme studies carried out on the islet tissue of fish may be extrapolated to mammalian islet tissues where direct enzyme study is difficult, we have compared the enzyme contents of corresponding tissues of the

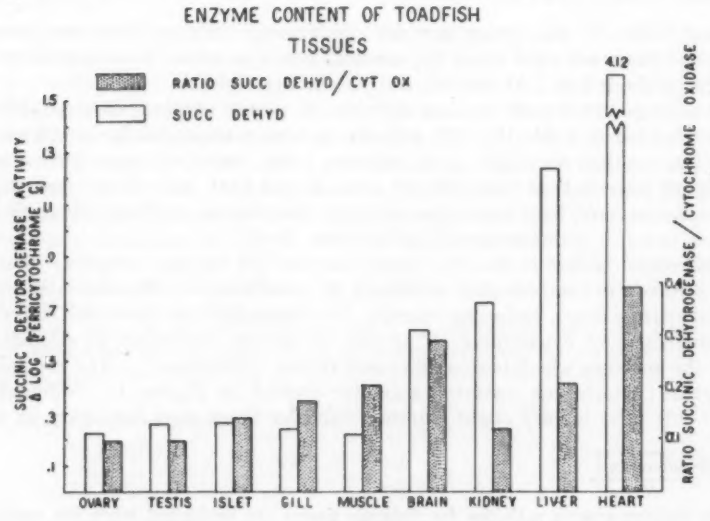

FIGURE 1.

rat and the toadfish. The enzyme activity of the most active tissue in each species (heart) is arbitrarily set at 100 per cent and the activity of the other tissues is expressed in terms of their relative activity (Table III).

Although the absolute enzyme activity of rat heart is about three times as great as toadfish heart, the relative enzyme concentrations in the corresponding tissues show good agreement in most cases. The toadfish kidney is an exception in that it contains only 18 per cent as much succinic dehydrogenase as toadfish heart, whereas rat kidney is 58 per cent as active as rat heart.

Discussion

Dunn, Sheehan and McLetchie showed in 1943 that alloxan selectively killed the beta cells in the islets of Langerhans of the pancreas. Although the exact mechanism by which alloxan destroys the pancreatic beta cells has remained obscure, it is apparent that alloxan is a selective rather than a specific poison, since larger doses

Table III

Relative enzyme content of tissues of toadfish and rat

	Cytochrome oxidase		Succinic dehydrogenase	
	Rat	Toadfish	Rat	Toadfish
Heart	100%	100%	100%	100%
Kidney	71	58	58	18
Liver	36	57	42	30
Brain	19	22	15	15
Muscle	12	11	6	6

of this compound will also produce necrosis of the liver and kidney cells (Dunn and McLetchie, 1943; Goldner and Gomori, 1943; Palay and Lazarow, 1946).

Several mechanisms have been suggested to explain the selectivity of alloxan for the beta cells (Lazarow, 1949). The metabolism of the beta cell differs from other cells in that it is continually synthesizing insulin. Insulin is a protein which has an unusually high content of sulfur amino acids (12 per cent cystine) (Du Vigneaud, 1927). Since all the sulfur of insulin is in the disulfide form, it might be expected that the metabolism of a cell synthesizing insulin would be geared to the oxidation of sulfhydryl groups to their disulfide form. Since sulfhydryl compounds such as glutathione and cysteine have been shown to protect rats against alloxan diabetes (Lazarow, 1946), and since cysteine is also used for the synthesis of glutathione, it has been postulated that the specialization of the beta cell for insulin synthesis may deplete the beta cell gluthathione and thereby increase the susceptibility of these cells to alloxan diabetes (Lazarow, 1949).

Alloxan is destroyed in the body by several reactions, one of which is its reduction to a non-diabetogenic derivative (Lazarow, Patterson and Levey, 1948), dialuric acid. This reduction, which can be accomplished by glutathione and by cysteine, can also be carried out by the reduced coenzyme, diphosphopyridine nucleotide (Cooperstein, Lazarow and Patterson, 1951). By contrast, dialuric acid can be re-oxidized to alloxan by cytochrome *c* (Cooperstein, Lazarow and Patterson,

1951) and by methylene blue (Lazarow and Liambeis, 1950). The potentiation of alloxan diabetes by methylene blue has been attributed to the reoxidation of any dialuric acid formed *in vivo* back to its diabetogenic derivative, *i.e.*, alloxan (Lazarow and Liambeis, 1950).

A further consequence of the specialization of the beta cells for insulin synthesis may be a decreased ability to destroy alloxan. If the metabolism of a cell is geared to the oxidization of sulfhydryl to disulfide, this cell may also be less able to reduce alloxan to dialuric acid (or it may rapidly reoxidize any dialuric acid formed back to alloxan). The finding by Bensley (1911) that islet tissue reduces Janus Green more slowly than does acinar tissue is in keeping with this hypothesis.

The enzyme studies reported here may likewise lend support to this hypothesis. Since dialuric acid is oxidized to an active diabetogenic compound, *i.e.*, alloxan, by the oxidized form of cytochrome *c*, it is apparent that the concentration of the oxidized form of this enzyme would play a role in determining the effective concentration of alloxan in the beta cell. The concentration of oxidized cytochrome *c* would in turn be determined by (a) the total concentration of cytochrome *c* present, by (b) the relative concentration of the enzymes which both oxidize and reduce cytochrome *c* and by (c) the spatial relations of these enzymes. Whereas the oxidation of cytochrome *c* is accomplished only by the cytochrome oxidase complex, the reduction of cytochrome *c* can be accomplished by a number of dehydrogenases. The succinic dehydrogenase complex is only one of the enzymes capable of reducing cytochrome *c*. It is apparent that both the absolute concentration of succinic dehydrogenase as well as the relative concentrations of succinic dehydrogenase to cytochrome oxidase in the beta cell would undoubtedly influence the fraction of cytochrome *c* that is in the oxidized state.

However, since other cytochrome-reducing systems would also play a role, it will be necessary to have a more complete picture of other enzymes in the islet tissue.

Since islet tissue was found to be low in succinic dehydrogenase and to have a low succinic dehydrogenase to cytochrome oxidase ratio (Fig. 1) (*i.e.*, it contains a relative abundance of cytochrome oxidase), it would appear that if the distribution of other cytochrome reducing enzymes parallels that of succinic dehydrogenase, islet tissue may well have a relatively high concentration of oxidized cytochrome *c* within the cell. Therefore, following the injection of a given dose of alloxan, any dialuric acid formed by the *in vivo* reduction of alloxan would be re-oxidized more readily and the effective concentration of alloxan within the beta cells would be higher. These cells might therefore be more susceptible to destruction by alloxan than would be the case with other tissues such as heart, liver, kidney, brain, and possibly muscle. On the basis of these enzyme studies, however, the gonads should be equally susceptible to alloxan. It must be remembered that on the other hand, the blood supply of an organ also determines the concentration of alloxan reaching that organ. The extremely rapid rate at which alloxan is destroyed does not permit equilibration to take place. On the basis of their blood supply, very vascular organs such as the islet tissue, liver, and kidney, might be expected to receive a higher total dose of alloxan than would be the case with the less vascular organs such as gonad or muscle.

It is of considerable interest that the relative enzyme activity of most of the tissues examined, using the enzyme content of the heart as 100 per cent, is the same

for rat and toadfish organs. This would suggest that the comparisons between the islet and other tissues of the toadfish may also reflect the enzyme status in mammalian tissue where direct analysis of islet tissue is more difficult. Toadfish kidney, however, differs from the other tissues studied in that it contains comparatively less succinic dehydrogenase than does rat kidney. The toadfish kidney also differs morphologically from rat kidney in that the former is aglomerular and it contains a large amount of lymphoid tissue. Whether this striking difference in enzyme content is a reflection of the differences in secretory mechanisms of glomerular and aglomerular kidneys, the presence of lymphoid tissues, the marine habitat, or the presence of gills which may take over a part of the renal excretory function, awaits further investigation.

Determinations of these enzymes in islet tissue with diminished numbers of beta cells (alloxan diabetic) will be carried out at a later date.

Summary

1. The succinic dehydrogenase and cytochrome oxidase contents of toadfish islet tissues have been determined by measuring spectrophotometrically the rate of reduction and oxidation of cytochrome *c* under standardized conditions.

2. The succinic dehydrogenase content of islet tissue was much lower than heart, liver, kidney or brain, and of the same order of activity as testis, ovary, muscle and gill.

3. The ratio of succinic dehydrogenase (a cytochrome *c*-reducing enzyme) to cytochrome oxidase (the cytochrome *c*-oxidizing enzyme) in islet tissues was low when compared to muscle, brain, liver and heart.

4. The relation of these findings to the selective destruction of the pancreatic beta cells by alloxan has been discussed.

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FURTHER STUDIES ON THE ANAEROBIC METABOLISM OF SOME FRESH WATER SNAILS [1]

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It has been shown (von Brand, Baernstein and Mehlman, 1950) that different species of fresh water snails show considerable differences in their resistance to anaerobic conditions. All species consumed carbohydrate anaerobically, but in contrast to what is known from mammalian tissues, lactic acid proved to be only a minor end-product of anaerobic carbohydrate breakdown in several species. It therefore appeared of interest to search for other metabolic end-products and in the present communication data are presented on the formation of volatile acids. This phase was selected because lower fatty acids have been found among the anaerobic metabolic end-products both of insects and worms (review of the literature in von Brand, 1946).

Material and Methods

The following species of snails were employed and where no further data are supplied, they were of the same derivation as stated previously (von Brand, Baernstein and Mehlman, 1950).

1. Pulmonates
   Planorbidae: *Australorbis glabratus, Helisoma duryi, Planorbarius corneus, Biomphalaria boissyi, Biomphalaria pfeifferi*
   Lymnaeidae: *Lymnaea palustris, Lymnaea natalensis*
   Physidae: *Physa gyrina, Aplexa nitens*
2. Operculates
   Thiaridae: *Melanoides tuberculatus*
   Amnicolidae: *Bulimus tentaculatus*, used shortly after being shipped from Lake Erie, Michigan

The general plan of the experiments was to incubate the snails anaerobically for the maximal period compatible with their anaerobic tolerance, to determine the carbon dioxide production during the period of incubation, and to establish at the end of this period whether any volatile acids had been excreted into the medium or accumulated in the tissues of the snails.

[1] Taken in part from a thesis submitted by B. Mehlman to the Department of Chemistry, Graduate School, Georgetown University, in partial fulfillment of the requirements for the degree of Master of Science.

[2] The authors wish to express their appreciation for the contribution of snails to Dr. E. G. Berry, Mrs. M. O. Nolan, and Dr. L. Olivier of this Laboratory and Dr. H. van der Schalie of the University of Michigan.

[3] Laboratory of Tropical Diseases.

The experimental procedure employed in anaerobic incubation and in determining the carbon dioxide production has been described previously (von Brand, Baernstein and Mehlman, 1950) and the same temperature, 30° C., was used. At the end of the experiments 1 ml. of the medium (dechlorinated tap water), *i.e.*, one-half the amount used initially, was withdrawn from each Warburg flask and the steam volatile acids were determined according to the procedure of Bueding (1949). The snails of each flask were immediately transferred to 0.5 ml. of 0.1 *N* NaOH and homogenized; 2.5 ml. of 0.45 per cent $ZnSO_4$ were added and adjusted to a total volume of 4 ml. The material was therefore deproteinized essentially according to Hagedorn and Jensen (1923). After centrifugation one-half of the supernatant was used for determination of the steam distillable acids.

After these series were concluded, attempts were made to identify the volatile acids. For these experiments only two species of snails, *Australorbis glabratus* and *Helisoma duryi*, were employed. As much medium as possible was removed from the flasks and the contents of six flasks were combined. Similarly, the snails of six flasks were used together and in some instances, as indicated, the medium and the snails were combined. The acids recovered by steam distillation were separated on silica gel columns according to the procedure of Ramsey and Patterson (1945). Confirmation of the chromatographic evidence was sought by applying to the separated acids some of the microchemical tests described by Klein and Wenzl (1932).

Results

The rate of carbon dioxide production of the various species of snails in most instances was in fairly good agreement with the values reported previously (von Brand, Baernstein and Mehlman, 1950), but in a few cases, especially *Lymnaea natalensis* and *Biomphalaria boissyi*, greater differences were obtained. No definite explanation can be suggested; it is possible that differences in size or possibly feeding conditions in the aquaria prior to the experiments played a role.

Insofar as details of the carbon dioxide production are concerned, data on two sets each of *Physa gyrina* and *Helisoma duryi* are presented in Figure 1. They are representative for the non-resistant and resistant group, respectively. It is obvious that the carbon dioxide production proceeded at a fairly regular, though slowly declining, rate. This is a significant point to which we will return in the discussion.

It is very probable that the carbon dioxide values presented are minimal values, since it appears likely that some carbon dioxide retention took place. It is obviously impossible to study this question with complete snails; their calcareous shells make such experiments impossible. We tried to approach the problem by homogenizing snails (*Australorbis glabratus*) after removing them from their shells and comparing the amounts of carbon dioxide liberated into the gas phase of Warburg vessels by a definite amount of lactic acid added through the sidearm with those liberated by the same amount of acid from a bicarbonate solution. We did find that the snail tissues retained per gram tissue about 200 $mm.^3$ carbon dioxide. We are not convinced, however, that this figure is correct; snail homogenates show a pH of about 8.3. It is by no means certain that the same pH would prevail in the tissues of intact snails, since it is possible that during the homogenizing process a loss of carbon dioxide occurs. There seems, at present, no practical way of eliminating this obvi-

ous source of error. In view of this situation we do not present this phase of our experiments in detail. It may be mentioned that the tissues of Australorbis have a considerable alkaline reserve; one gram of tissue liberated upon acidification to pH 3.9 about 2270 mm.^{3} of carbon dioxide.

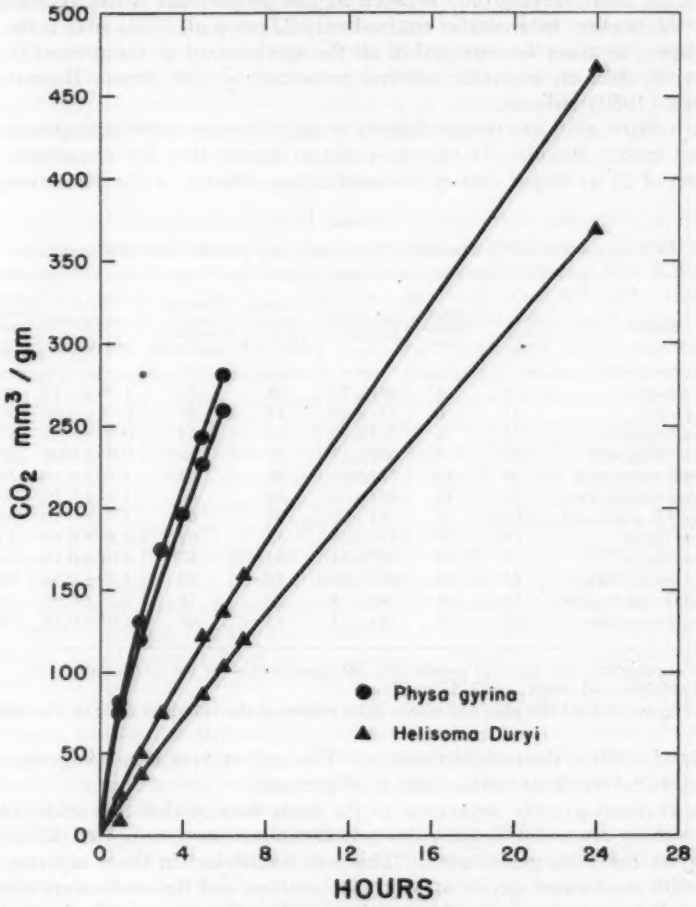

FIGURE 1. Carbon dioxide production of *Physa gyrina* and *Helisoma duryi* in relation to the length of the anaerobic period.

It was established that the snails did not have volatile acids in their tissues before they were subjected to anaerobic conditions. Control determinations with snails taken directly from the aquaria were done with each species and yielded completely negative results. After being exposed to anoxic conditions, on the contrary, significant amounts of volatile acids were found in every case both in the medium and

within the tissues (Table I). It should be noted that the species with little anaerobic resistance (*Aplexa nitens, Physa gyrina, Lymnaea palustris* and *Lymnaea natalensis*) retained a somewhat larger percentage of the acids in their tissues than the resistant ones. In most of the latter surprisingly close agreement was found, the retained acids varying only between 32 and 39 per cent of the total acids produced. *Melanoides tuberculatus* retained only 22 per cent. This snail is the species best adapted to anaerobic survival of all the species used in the present investigation, as the data on anaerobic survival presented by von Brand, Baernstein and Mehlman (1950) indicate.

The volatile acids are unquestionably a major source of the manometrically determined carbon dioxide. In the non-resistant species they are responsible for the evolution of 21 to 40 per cent of the total carbon dioxide, while the corresponding

TABLE I

CO_2 and volatile acid production of fresh water snails under anaerobic conditions

Species	No. of exper.	Anaerobiosis hours	Total CO_2 mm.³/gm.	Volatile acid in tissue % total acid	Volatile acid in medium % total acid	Total volatile acid in ml. of .005 *N*/gm.	% total CO_2 liberated from inorganic source by volatile acid
Aplexa nitens	18	6	381±23	48	52	1.39±0.12	40±2
Physa gyrina	12	6	272±10	46	54	0.78±0.06	32±2.4
Lymnaea palustris	21	6	262±20	56	44	0.50±0.05	21±1.3
Lymnaea natalensis	15	6	400±17	44	56	0.93±0.09	26±2.1
Biomphalaria boissyi	24	16	772±27	36	64	3.76±0.15	55±1.9
Biomphalaria pfeifferi	22	16	402±25	34	66	1.94±0.10	55±1.6
*Australorbis glabratus**	23;30	16	633±29	39	61	3.99±0.19	69±3.3
Helisoma duryi	15	24	436±23	38	62	2.88±0.19	72±2.9
*Helisoma duryi***	17	24	547±24	33	67	4.01±0.18	82±3.1
Planorbarius corneus	13	24	601±50	34	66	3.20±0.34	58±2.8
Melanoides tuberculatus	16	24	140± 8	22	78	0.46±0.06	35±2.8
Bulimus tentaculatus	16	24	255±24	32	68	2.07±0.18	90±3.2

* 23 experiments for the acid production, 30 experiments for the CO_2 production.

** Penicillin and streptomycin in medium.

The figures behind the plus and minus signs represent the standard error of the mean.

range is 55 to 90 in the resistant species. The only exception in this group is again *Melanoides tuberculatus*, with a value of 35 per cent.

The chromatographic separation of the acids showed that two acids were produced both by *Australorbis glabratus* and *Helisoma duryi*, only two distinct bands forming on the silica gel columns. This was established in three separate experiments with the former species in which the medium and the snails were used separately and one experiment in which medium and snails were combined. In the case of *Helisoma duryi*, lack of material prevented a separate study of medium and tissues; two combined samples were investigated. In every instance, the threshold values of the acids corresponded closely to those of acetic and propionic acids and titration of the fractions gave for both species an approximate ratio of 2:1 for the acetic and propionic acid portions, respectively. This was true in the case of Australorbis, both for the medium and the tissues.

The propionic fraction recovered from Australorbis gave with mercurous nitrate

typical crystals of the mercury salt microscopically indistinguishable from those prepared from pure propionic acid. In the case of Helisoma, the copper salt was prepared; the amounts were small, but deep blue crystals characteristic of the copper salts of lower fatty acids were obtained and they were soluble in alcohol. It seems probable, then, that we were dealing in both cases with propionic acid.

The acetic acid fractions gave with mercurous nitrate only non-characteristic crystals. This happens frequently when the slightest traces of impurities are present (Klein and Wenzl, 1932). With copper sulfate deep blue crystals were also obtained and they showed the typical insolubility in alcohol. There can hardly be a doubt, therefore, that we were dealing with acetic acid.

Discussion

The present investigation has shown that snails kept under anaerobic conditions produce volatile acids, which they partly excrete into the medium and partly retain in their tissues, the acids involved being acetic and propionic acids. The formation of the former of these acids is not too surprising since acetic acid is a rather frequent end-product of anaerobic carbohydrate utilization both in bacteria and some higher organisms. Propionic acid, on the other hand, has been found only in the case of Ascaris insofar as multicellular organisms are concerned. Since our snails were not bacteriologically sterile, the question immediately arises whether the fatty acids were actually produced by the snail tissues or were due to bacterial activity which conceivably might have transformed lactic acid derived from the anaerobic metabolism of the snails into the lower fatty acids. It is well known that this question gave rise to a prolonged controversy in the case of parasitic worms. It has recently been decided unequivocally by Epps, Weiner and Bueding (1950), who succeeded in sterilizing *Ascaris lumbricoides* by means of antibiotics and found that these worms did produce lower fatty acids, among them acetic and propionic acid.

We carried out a series of experiments with *Helisoma duryi* kept in dechlorinated tap water containing 2000 units of penicillin and 10,000 units of streptomycin. As the data of Table I show, these snails produced somewhat more carbon dioxide and volatile acid than the nontreated snails, but even this medium yielded upon transfer to a nutritive medium and subsequent incubation some bacterial colonies, and there is no justification to assume that the alimentary tract of the snails would have been sterilized. We abandoned further attempts to sterilize snails because we gained the impression that prolonged stay in the antibiotic solutions, though not killing the snails, affected them adversely.

The Australorbis used in the chromatography experiments were laboratory reared, while the Helisoma were freshly collected from large tanks in Kenilworth Gardens, Md. Both nevertheless yielded the same acids; if bacterial activity was involved, both species would presumably have had to carry the same bacterial flora. Both species also accumulate acids in their body. The internal tissues of snails are presumably sterile; one would then have to assume that the metabolic end-products would have been transported first to contaminated organs, the alimentary tract and perhaps the outside tissues, and have been transformed there to fatty acids. These assumptions, although not impossible, are none too probable. It should also be remembered that the carbon dioxide production of the anaerobic snails declined somewhat with time; if a bacterial population would have been built up, one would have

expected rather an increase in rate. We are inclined to assume tentatively that the acids originated from the metabolism of the snails, but we concede that the final decision will have to wait for work on bacteriologically sterile snails.

We wish to emphasize strongly, however, that the biological implications of our findings are unaffected by the question of whether the snails themselves produced the volatile acids or not, since in nature no sterile snails can be expected to occur. We have pointed out previously (von Brand, Baernstein and Mehlman, 1950) that the non-resistant species, in contrast to the resistant ones, accumulated lactic acid within their tissues and we assumed that this difference may be one of the factors explaining the difference in resistance to anaerobiosis. We pointed out, however, that lactic acid could not be responsible alone for the death of the snails, because the resistant species also ultimately died of asphyxiation. Our present results would seem to indicate that the marked accumulation of volatile acids in the tissues may

TABLE II

Percentage of anaerobic carbon dioxide liberated from inorganic sources

Species	Per cent of total carbon dioxide liberated from inorganic sources		
	By lactic acid*	By volatile acids	Total
Aplexa nitens	67	40	107
Physa gyrina	38	32	70
Lymnaea natalensis	139	26	165
Lymnaea palustris	41	21	62
Biomphalaria boissyi	25	55	80
Biomphalaria pfeifferi	17	55	72
Australorbis glabratus	13	69	82
Helisoma duryi	4	72	76
Planorbarius corneus	3	58	61
Melanoides tuberculatus	17	35	52

* Data from von Brand, Baernstein and Mehlman (1950).

have a bearing on the problem. The lower fatty acids are probably less toxic than lactic acid, but still if they accumulate to a certain level they may be harmful. The difference in the respective amounts of end-products of various toxicities within the tissues of different species of snails may well be at the root of the differences in anaerobic resistance. Whether it is the sole factor involved cannot be stated with any degree of certainty, however.

It would obviously be premature to speculate about the mode of formation of the volatile acids, but a brief discussion of the carbon dioxide picture seems appropriate. We have pointed out in our previous paper that the carbon dioxide evolved by the non-resistant species is largely of direct inorganic origin, that is, liberated by lactic acid from bicarbonate. The relevant data concerning this point are shown again in Table II. The third column shows the amounts of carbon dioxide liberated from inorganic sources by the volatile acids. It is now clear that in all species a very large percentage of the carbon dioxide is of direct inorganic origin. It is possible or even probable that the remainder of the carbon dioxide unaccounted from this source is true respiratory carbon dioxide, carbon dioxide derived from the break-

down of carbohydrate. No accurate data along this line can be calculated, however, because of our inability to elucidate adequately the question of carbon dioxide retention by the tissues. It may be pointed out that this source of error may be responsible for the fact that less carbon dioxide was found in the cases of *Lymnaea natalensis* and *Aplexa nitens* than corresponds to the acids produced.

Summary

1. Fresh water snails exposed to anaerobic conditions produce volatile acids which are partly excreted into the medium and partly accumulate in the tissues.

2. The acids formed by *Australorbis glabratus* and *Helisoma duryi* were identified by chromatographic means and crystallographic data as propionic and acetic acids.

3. While bacterial formation of these acids cannot be excluded categorically, some evidence is adduced to the effect that they may be produced by the snail tissues.

4. The evidence indicates that the species not resistant to anaerobiosis are killed primarily by the accumulation of lactic acid, while the resistant species are more tolerant to the lack of oxygen due to the fact that they accumulate in their tissues the less toxic fatty acids rather than lactic acid.

5. Most of the carbon dioxide evolved by anaerobically kept snails is of direct inorganic origin.

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THE CHAETOGNATHA OF THE WEST COAST OF FLORIDA[1]

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Very little work has been undertaken on the Chaetognatha of the Gulf of Mexico. A short publication by Ritter-Zahony (1910) on specimens collected in the region of the Dry Tortugas, Florida, is the single paper available dealing directly with chaetognaths which might be considered as inhabitants of the Gulf. Conant (1895, 1896) has published notes and descriptions of species from the West Indies and Beaufort, North Carolina, representatives of which are found in the Gulf plankton. Davis (1949) and King (1949) have published preliminary reports on the plankton along the coast of Florida which include brief mention of the chaetognaths. Certain species of chaetognaths found in the Gulf have a cosmopolitan distribution. Reference to these species collected in other regions may be found in many papers.

The purpose of this study was to determine the species present along the west coast of Florida, their distribution, abundance and breeding seasons through an annual cycle. From previous sampling it was known that an appreciable gradient in salinity extended from the coastline to 15 miles or more offshore. It was desirable, therefore, to set up stations within this zone of variable salinity and to determine its effects on chaetognath distribution. Moreover, by attempting to locate the stations in the same relative salinity a truer comparison could be made between the populations of chaetognaths at different points along the coast.

Beginning in November, 1948 and continuing through January, 1950 plankton collections were made at monthly intervals from three well-spaced areas along the west coast. Ft. Myers Beach, Bradenton Beach and Cedar Key were selected as base points and from each of these bases three collecting stations were established approximately ½, 5 and 15 miles offshore (Fig. 1). For convenience in reference in this study, the bases are indicated by the numbers 1 (Ft. Myers Beach), 2 (Bradenton Beach) and 3 (Cedar Key), and the stations by A, B, and C from inshore to offshore, respectively. Thus, the inshore station at Ft. Myers is indicated by 1A and the offshore station at Cedar Key by 3C. These stations were all located on the inner border of the continental shelf which on the west coast of

[1] I am indebted to the University of Florida for providing funds which made this work possible. Dr. Ralph A. Morgen, Director of the Engineering and Industrial Experiment Station and Dr. C. Francis Byers, Professor of Biology, cooperated in the organization of this project. Mr. J. M. Thomson, Research Officer, Fisheries Research Station, Dunwich, Queensland, Australia, sent me identified specimens of chaetognaths taken from Australian waters. Dr. J. H. Fraser, Marine Laboratory, Aberdeen, Scotland, examined a number of specimens sent to him for identification and offered several useful suggestions. Dr. Fenner A. Chase, Jr., Curator, Division of Marine Invertebrates, United States National Museum, allowed me to work in the museum and to examine the identified chaetognaths stored there. Mr. E. D. McRae aided in the collection of the samples. I am grateful to Miss Esther Coogle, staff artist, for the careful drawings in this paper.

Part of the cost of publication was furnished by the University of Florida.

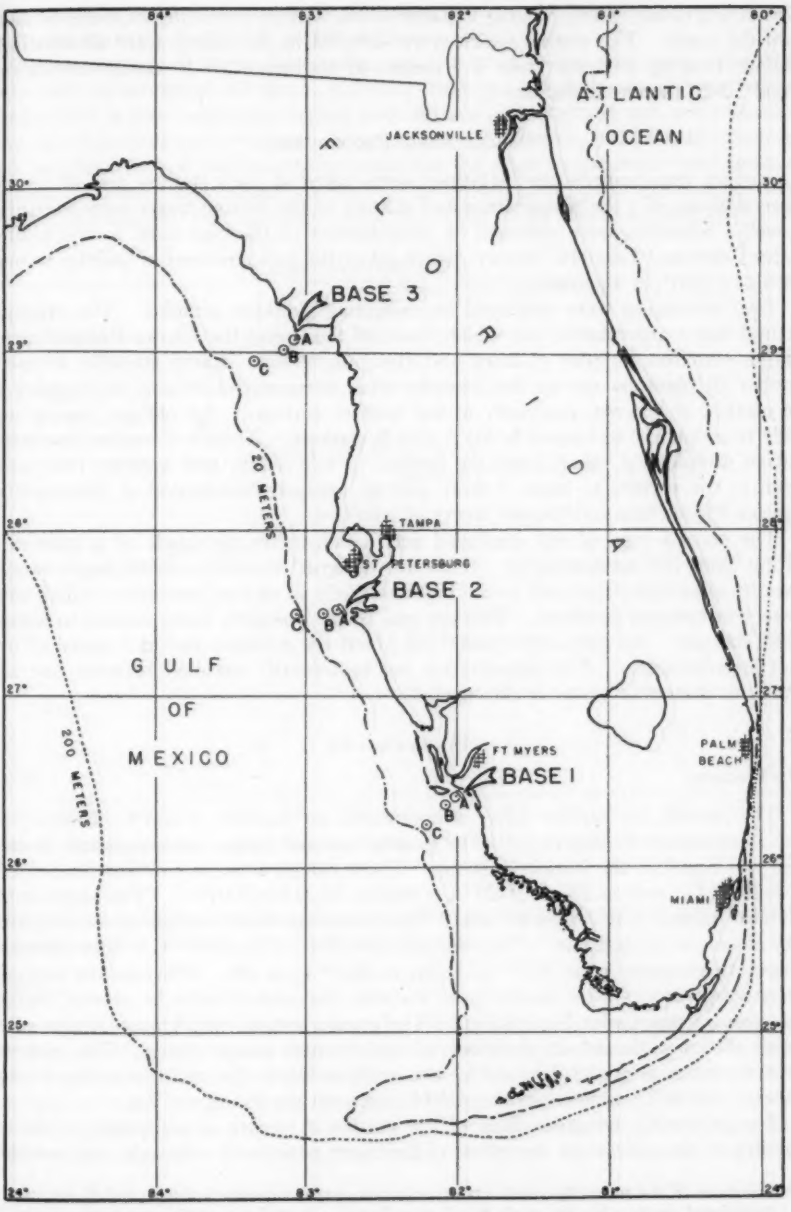

FIGURE 1. Map of Florida with the bases and stations on the west coast indicated.

Florida is a broad, gently sloping expanse of sea bottom extending 75 miles or more from the coast. The waters which were sampled in this study were all relatively shallow, ranging in depth from 4–5 meters at stations A, 6–10 meters at stations B and 12–18 meters at stations C.

Collecting Procedures

Surface temperatures and salinities were taken at each station, and at all offshore stations (C) the temperature and salinity of the bottom water were measured as well. Salinities were obtained by hydrometers of the type used by the United States Coast and Geodetic Survey, which allow the measurement of salinity to one-tenth of a part per thousand.

Two techniques were employed for collecting plankton samples. The principal method was a quantitative one which involved the use of the Clarke-Bumpus quantitative plankton sampler (Clarke and Bumpus, 1940). Early attempts to use a number 20 plankton net on this sampler were unsuccessful because of clogging of the meshes of the net, especially at the inshore stations. An oblique towing was made from surface to bottom at all A and B stations. At each C station one quantitative sample was taken from the bottom to mid-depth, and another from mid-depth to the surface to learn if there was an unequal distribution of chaetognaths between the surface and bottom layers of water.

The second type of net employed was a half-meter net made of a number 6 bolting cloth (74 meshes/inch). This was designed to catch a much larger sample than the quantitative net and would be more likely to capture specimens which were present in reduced numbers. This net was towed obliquely from surface to bottom at all stations. All nets were pulled for about ten minutes and at a speed of one knot approximately. The quantitative net customarily sampled between one and two cubic meters of water in this period.

Hydrography

Temperature

The records for surface water temperatures for stations A and C of bases 1, 2 and 3 are shown in Figure 2.[2] The greatest annual range in temperature at each base was found at the inshore stations. These ranges were as follows: station 1A, 19.8–29.7° C.; station 2A, 19.9–29° C.; station 3A, 15.9–29.6° C. The range at the offshore stations was somewhat less. The maximum water temperatures compared very closely at all stations. They varied from 29.0° C. to 29.9° C. The minimum temperatures varied from 15.9° C. at 3A to 20.8° C. at 1B. Whereas the warmest water temperatures were similar at all stations, the coldest water by several degrees was found at the Cedar Key station. The greater annual temperature range noted at this station is the result, therefore, of cooler water temperatures. The temperatures recorded at Cedar Key (3A) are comparable to the records of the United States Coast and Geodetic Survey (1944) taken in the vicinity of 3A.

The noticeably irregular lines of the graphs in winter as compared to the remainder of the year show the effect of the short periods of relatively cold weather

[2] Because of the normally small variation in temperature between the A and C stations, it was considered inadvisable to graph the figures for the B stations which were intermediate.

which normally occur in this area in the winter. The shallow inshore waters respond rather quickly to chilling by the cold air.

The temperature of the surface water and the water about one meter from the bottom was measured at all the C stations. Bottom temperatures in most cases agreed within a few tenths of a degree with surface temperatures and were usually cooler by a few tenths of a degree. In a number of instances, however, the surface water was the cooler of the two or the water was the same temperature from surface to bottom. These observations, as well as the information received from salinity records and plankton counts, indicate that the water from shore, at least as far as

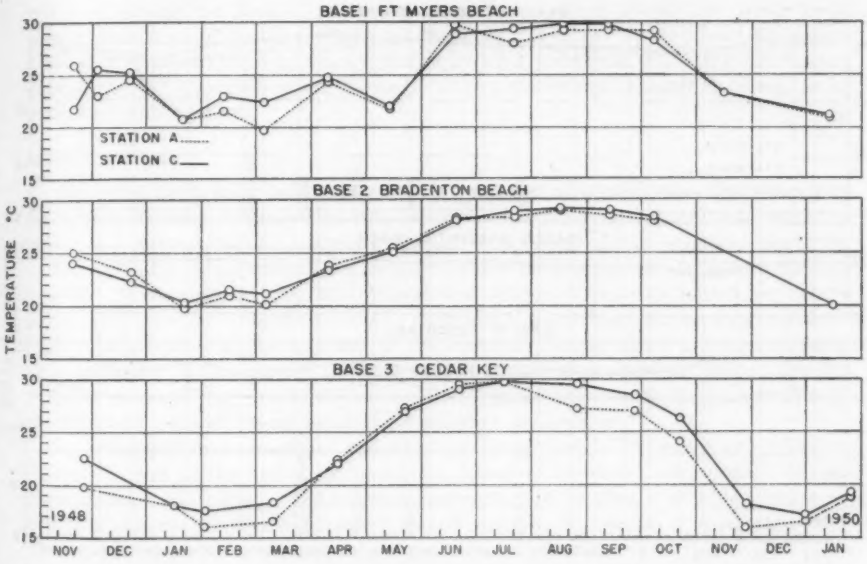

FIGURE 2. Monthly surface water temperatures at all A and C stations. Temperatures at the B stations were intermediate and were omitted to prevent confusion.

the C stations, is almost continuously undergoing mixing and that little stratification is allowed to take place at any season of the year.

Salinity

An inspection of Figure 3 shows that the salinity of the inshore waters is notably more reduced than that of the offshore stations because the run-off from the land causes appreciable dilution of the shallow coastal water. Bar graphs of rainfall have been included for Ft. Myers Beach and Cedar Key to show the direct effects of rainfall on salinity at stations 1A and 3A.[3] The range in salinity is much greater at the inshore stations. This is less noticeable at station 2A because it was rela-

[3] U. S. Dept. of Commerce, Weather Bureau. 1948–50. Climatological Data. Florida. Vol. 52, No. 13, Vol. 53 and Vol. 54.

tively farther away from the mainland drainage than either 1A or 3A. The lowest salinities were usually at 3A and were in part the result of the outflow of the neighboring Suwannee and Waccasassa Rivers in addition to the run-off from many smaller streams and marshes in that area. The highest salinities were found at stations 1C and 2C and compare closely with salinities encountered on one collecting trip which extended about 40 miles offshore in the Bradenton Beach area. The effects of dilution on salinity were noticeable at 3C.

The effects of the tide on salinity were measured at station 3A on three occasions between successive high and low tides. The salinity range in these cases was

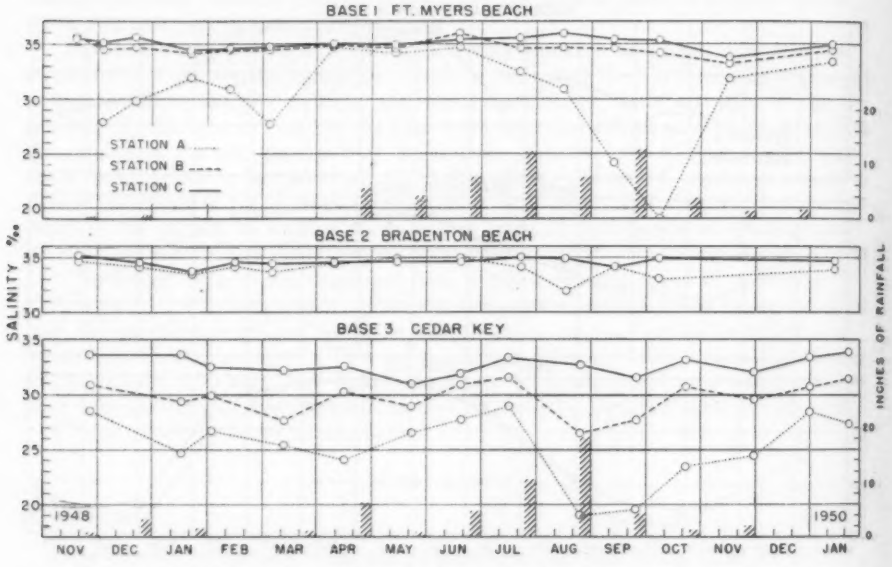

FIGURE 3. Monthly surface salinities. The bar graphs indicate total monthly rainfall at Ft. Myers and Cedar Key.

1.5°/₀₀, 1.9°/₀₀, 3.5°/₀₀. At all inshore stations these variations with the tide are undoubtedly responsible for some irregularities in the curves of salinity; however, they do not account for the more pronounced trends which are dependent to a large extent on rainfall.

MEASUREMENTS AND MATURITY STAGES

Measuring and staining

All chaetognaths were measured to the nearest millimeter. Measurements of length were made exclusive of the caudal fin. In order to see in detail certain features of the anatomy, especially the corona and gonads, it is frenquently necessary to stain the specimens. A convenient stain for this purpose is a one per cent solution of methylene blue in which the specimen need be immersed only a few

minutes (Thomson, 1947). A more permanent stain which is effective for the ovaries and testes is alum carmine. The gonads are readily visible in specimens which have been left in a weak solution of this stain for several hours or overnight. If the tissues become too deeply stained, they may be de-stained rapidly by immersing the specimen in a few milliliters of water to which has been added a drop of hydrochloric acid.

It was found that by placing a chaetognath in a 2 or 3 per cent solution of iodine and potassium iodide for a few minutes, its teeth and hooks, ordinarily stain resistant, became slightly stained. This is an aid in counting the teeth when the chaetognath is held against a white background. The iodine, moreover, has the effect of giving the flesh a putty-like consistency which allows the teeth to be pressed into a more visible position for counting. The use of the above technique, coupled with observation of the specimens under a wide-field dissecting microscope capable of 100 × magnification, normally allows specimens as small as 5 mm. to be examined with success.

Maturity stages

Several criteria have been suggested by a number of investigators for separating the hermaphroditic chaetognaths into stages which represent progressive states of maturity. Kramp (1917) initially proposed recognition of four stages of maturity. Russell (1932) reduced Kramp's stages to three as a result of studies on *Sagitta elegans*. Wimpenny (1936) has defined four stages of maturity which are rather similar to Russell's. Thomson (1947) proposed a subdivision of maturity into four groups based only on development of the ovaries. While there is a considerable basic similarity in the various systems proposed, attention must be paid to the description of each stage specified by an investigator because it is not necessarily identical with similarly numbered stages of other workers.

In this paper three stages of maturity are recognized. These are as similar to Russell's stages as the individual variations found in the developing gonads of different species will allow. Three stages are sufficient to give a reliable picture of breeding and the inclusion of more stages adds to the work of separating the chaetognaths without commensurate returns in information.

In this problem either the entire sample or a fraction, usually containing 30–40 chaetognaths, was taken from the collection made at each station. All the specimens selected were measured, stained and graded into maturity stages. Because a very appreciable difference may be observed in the state of maturity for any given body length, it is believed that more valid conclusions may be reached by this procedure than by comparing the length and stage of maturity from a limited number of specimens and assuming that the remainder of the sample can be divided into maturity stages on the basis of length measurements alone.

The stages of maturity recognized in this study are:

Stage I. Immature chaetognaths which show no clusters of maturing sperm cells or spermatozoa in the tail cavity. Ovaries small or indistinct.

Stage II. Clusters of sperm cells in tail cavity. Ovaries show signs of growth. No enlarged eggs present at this stage.

Stage III. Ovaries have increased in length and some of the individual eggs have increased appreciably in size.

There is a considerable difference in the shape and size of the ovaries and in the size of the mature ova. In the case of two of the species collected, *Sagitta hispida* and *S. helenae*, the lengthening of the ovary was pronounced in the mature forms, whereas the increase in size of the individual eggs was gradual, the eggs never

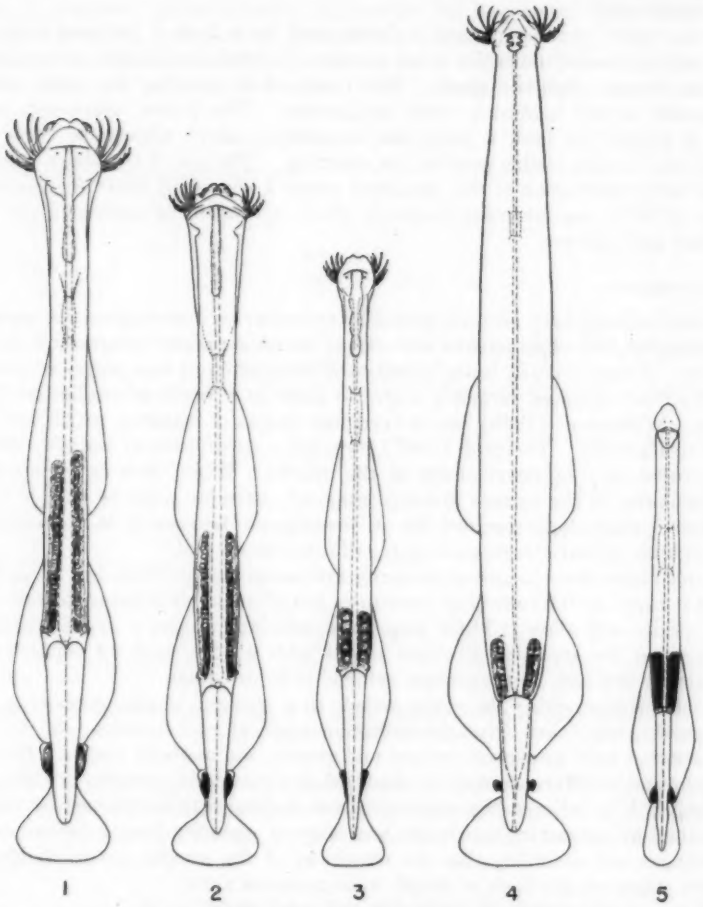

FIGURE 4. The species of chaetognaths collected along the west coast of Florida. 1. *S. hispida* 12×; 2. *S. helenae* 6×; 3. *S. tenuis* 12×; 4. *S. enflata* 6×; 5. *K. pacifica* 12×.

becoming very large. It was therefore decided to define the beginning of Stage III for these species as that point at which the lengthening ovary reached as far forward as the posterior end of the anterior fin. In the remaining species collected the ovaries never extended very far forward in the body cavity, but the size of the

developing eggs was more evident and this was the criterion used to define Stage III as indicated above.

The differences in the details of the development of the gonads of the various species of chaetognaths are very noticeable. Not only are there differences in rate of development and size of the ovaries, but in the case of *S. enflata*, for example, the sperm balls which normally circulate freely in the tail cavity become concentrated in two packed masses at the extremity of the tail segment.

West Coast Chaetognaths

List of species. Two genera and five species of chaetognaths were collected from the west coast of Florida during the course of this investigation. These were: *Sagitta hispida* Conant, 1895; *Sagitta helenae* Ritter-Zahony, 1910; *Sagitta tenuis* Conant, 1896; *Sagitta enflata* Grassi, 1881; *Krohnitta pacifica* (Aida), 1897.

Sagitta hispida Conant (Fig. 4)

Sagitta hispida Conant, 1895, p. 290, 1896, p. 214; Fowler, 1906, p. 58; Michael, 1911, p. 27, 1919, p. 239; Burfield and Harvey, 1926, p. 96.

Sagitta robusta Ritter-Zahony, 1910, p. 136.

Description. *S. hispida* was originally but incompletely described by Conant (1895) from specimens obtained at Beaufort, North Carolina. This species has

Table I

Measurements of S. hispida from the west coast of Florida

Length mm.	Tail segment %	Hooks	Anterior teeth	Posterior teeth
4–5	26–27	6–8	4–6	10–11
6–7	27–28	7–9	5–6	7–10
8–9	26–29	7–8	6–9	10–12
10–11	26–29	7–8	6–9	10–12
12–13	26	8	7	10

been mis-identified on more than one occasion in the literature. Aida (1897) reported it from the harbor of Misaki in Japan. Tokioka (1939), who has studied the chaetognath fauna of Japan extensively, synonymizes *S. hispida* Aida with *S. robusta* Doncaster. Burfield and Harvey (1926) reported *S. hispida* from the Indian Ocean but their statement that the posterior fins do not extend to the seminal vesicles and that there is slightly more fin on the trunk than on the tail is not in agreement with Conant's figure or my data.

There are three specimens of *S. hispida* in the United States National Museum, which were deposited there in 1896 by F. S. Conant (Cat. No. 4984).[4] The dental formulae could not be determined without damaging these specimens, all of which were not in good condition when examined; however, the outline of the body, the position and shape of the fins and the seminal vesicles could be seen clearly on one, and these features agreed with Conant's (1895) figure and description for this

[4] Through the courtesy of Dr. Fenner A. Chase, Jr., Curator, Division of Marine Invertebrates, I was allowed to examine these specimens.

TABLE II

Measurements of S. hispida

Length mm.	Tail segment %	Hooks	Anterior teeth	Posterior teeth
Three specimens from Beaufort Inlet, N. C.[5]				
5	29	7	4	8
7	29	6	6	9
8	27	6	6	9
Data from Conant, 1895, Beaufort, N. C.				
7–11	33	8–9	4–5	8–15

TABLE III

The occurrence of chaetognaths at Base 1

Figures represent numbers per cubic meter

Species at Stations A, B, C, C'*

Date	*S. hispida*				*S. helenae*				*S. tenuis*				*S. enflata*				*K. pacifica*			
	A	B	C	C'	A	B	C	C'	A	B	C	C'	A	B	C	C'	A	B	C	C'
1948																				
19 Nov.	111	0	–	–	0	48	–	–	40	11	–	–	7	3	–	–	3	22	–	–
22 Dec.	10	0	0	0	0	48	6	7	10	28	13	41	0	34	83	45	1	11	6	0
1949																				
21 Jan.	2	99	158	48	0	4	4	3	2	106	6	8	0	0	19	20	0	2	3	0
12 Feb.	6	49	0	0	0	1	47	44	0	19	6	19	0	1	28	19	0	2	0	2
4 Mar.	41	15	0	0	0	0	45	36	41	37	7	25	0	0	24	67	0	0	3	0
9 Apr.	78	53	0	0	0	0	53	100	7	117	10	7	0	11	13	4	0	0	0	0
13 May	197	33	0	0	0	0	96	116	0	2	24	0	0	0	28	19	0	0	5	5
18 June	23	68	0	0	0	0	325	197	0	5	8	9	0	0	17	17	0	0	0	0
21 July	52	18	0	0	0	0	32	20	0	0	0	0	0	0	0	1	0	0	0	0
17 Aug.	41	73	–	0	0	0	–	25	0	0	–	0	0	0	–	0	0	0	0	0
12 Sept.	141	77	44	56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7 Oct.	46	44	0	0	0	0	34	30	0	0	0	0	0	0	0	3	0	0	0	0
15 Nov.	130	35	0	–	0	17	31	0	4	0	0	–	0	78	6	–	0	28	0	–

* C net towed from 0 to 8 meters, C' net towed from 8 meters to bottom. – Indicates absence of sample at that station.

species. Conant's use of a small specimen with the hood covering the head gives the impression that the head is distinctly narrower than it appears when the hooks are extended and the hood drawn back. As indicated in the table of measurements, a few specimens of *S. hispida* were secured from the entrance to Beaufort Harbor, the type locality.[5] These are in essential agreement with Conant's specimens and with the material which has been collected from the west coast of Florida.

[5] Specimens supplied through the courtesy of Dr. W. H. Sutcliffe, Jr., Duke Marine Laboratory, Beaufort, N. C.

S. hispida is a smaller than average chaetognath. The size at maturity ranges between 5 and 12 mm., 8 mm. being common. The body is rigid, collarette conspicuous, corona elongate, extending from a point anterior to the eyes well down on the body, but not reaching as far as the ventral ganglion. The fins are completely supplied with rays. The posterior fin touches the seminal vesicles in mature specimens. The number of anterior teeth is variable, ranging from 4–6 in small forms to as many as 9 in the larger specimens. These teeth are held flat against the head, a feature which in addition to the smaller number allows this species to be distinguished from *S. helenae*.

TABLE IV

The occurrence of chaetognaths at Base 2

Figures represent numbers per cubic meter

Species at Stations A, B, C, C′*

Date	*S. hispida*				*S. helenae*				*S. tenuis*				*S. enflata*				*K. pacifica*			
	A	B	C	C′	A	B	C	C′	A	B	C	C′	A	B	C	C′	A	B	C	C′
1948																				
20 Nov.	–	18	0	–	–	11	77	0	–	6	11	–	–	0	23	–	–	0	11	–
23 Dec.	–	22	0	0	–	1	26	3	–	1	18	16	–	0	25	13	–	0	8	1
1949																				
22 Jan.	15	0	0	0	0	15	4	9	7	4	3	9	0	4	9	88	0	0	0	2
13 Feb.	57	11	0	0	0	0	15	28	4	0	12	34	0	4	91	333	0	0	0	0
5 Mar.	33	0	0	0	0	0	11	7	14	0	0	0	0	0	8	32	0	0	0	0
10 Apr.	12	27	0	0	0	10	11	8	12	14	7	26	0	0	2	11	0	12	0	0
14 May	45	15	0	0	0	5	4	14	5	9	0	1	0	0	4	10	0	0	0	0
19 June	46	7	0	0	0	18	46	75	0	0	5	13	0	0	2	8	0	0	0	0
22 July	9	24	0	0	0	0	23	0	0	6	0	0	0	0	0	0	0	0	0	0
16 Aug.	54	0	0	0	0	58	50	25	0	2	0	0	0	0	0	0	0	0	0	4
13 Sept.	112	0	0	0	0	15	45	29	4	3	3	7	0	0	0	2	0	0	3	2
7 Oct.	29	17	21	69	0	2	0	19	6	2	0	0	0	0	2	0	0	8	1	19
1950																				
Jan.	49	4	0	–	0	2	32	–	4	3	11	–	0	3	9	–	0	1	1	–

* C net towed from 0 to 8 meters, C′ net towed from 8 meters to bottom. – Indicates absence of sample at that station.

Distribution. This species was taken consistently at all the A stations. A comparison of Tables I, II, III and Figure 3 shows the direct relationship of this species with the shallow, less saline coastal water. No immediate connection could be seen, however, between the month to month fluctuation of salinity at any one station and the appearance or disappearance of *S. hispida* at that station. Moreover, despite the similarities in the salinity at stations 2A and 2C, this species was almost always abundant at the inshore station (2A) and with one exception was never taken at the offshore station (2C). This suggests that some factors in addition to salinity are operating to control the distribution of chaetognaths in this area. It does not appear likely that temperature exerts any appreciable effect; for example, compare the similar temperatures at 2A and 2C (Fig. 2).

In addition to its occurrence at the regular stations, *S. hispida* was collected just off the tip of Cape Sable, August, 1949; from a point midway along the Florida Keys (Bow Channel, Sugarloaf Key) January, 1947; a channel one mile north of Key West, June, 1946; and, in company with *S. helenae*, at a point about 12 miles northwest of Key West, July, 1946. These records further indicate its preference for inshore sea water of reduced salinity.

Breeding. In the warm waters of the Gulf, *S. hispida* breeds to some extent the year round (Table IV). There was no month in which a high percentage of

TABLE V

The occurrence of chaetognaths at Base 3

Figures represent numbers per cubic meter

Species at Stations A, B, C, C'*

Date	*S. hispida*				*S. helenae*				*S. tenuis*				*S. enflata*				*K. pacifica*			
	A	B	C	C'	A	B	C	C'	A	B	C	C'	A	B	C	C'	A	B	C	C'
1948																				
26 Nov.	–	57	0	0	–	0	21	27	–	0	8	0	–	0	3	0	–	0	0	0
Dec.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1949																				
16 Jan.	–	–	–	0	–	–	–	80	–	–	–	0	–	–	–	88	–	–	–	0
2 Feb.	18	27	122	88	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12 Mar.	46	28	0	0	0	0	31	71	0	0	11	28	0	1	2	4	0	0	0	0
15 Apr.	52	37	0	16	0	0	10	16	4	7	24	64	0	0	0	5	0	0	0	0
22 May	223	77	0	0	0	0	53	95	0	8	1	2	0	0	3	0	0	0	0	0
19 June	481	106	14	47	0	0	14	31	0	2	20	25	0	0	0	0	0	0	0	0
15 July	53	45	15	23	0	0	0	0	0	1	15	4	0	0	2	0	0	0	0	0
24 Aug.	–	37	8	17	–	0	2	0	–	0	2	2	–	0	0	0	–	0	0	0
25 Sept.	–	160	62	24	–	0	0	0	–	0	0	0	–	0	0	0	–	0	0	0
22 Oct.	–	94	22	31	–	0	0	0	–	0	0	0	–	0	0	0	–	0	0	0
29 Nov.	21	2	–	–	0	0	–	–	3	0	–	–	0	0	–	–	0	0	–	–
31 Dec.	–	13	0	–	–	0	22	–	–	0	1	–	–	0	2	–	–	0	1	–
1950																				
Jan.	13	2	2	–	0	0	17	–	–	0	8	–	–	0	1	–	–	0	1	–

* C net towed from 0 to 8 meters, C' net towed from 8 meters to bottom. – Indicates absence of sample at that station.

Stage I individuals were not present. In March and July, 11 per cent were mature Stage III forms. There is some evidence, judging by the increase in young as well as by the appearance of mature specimens, that reproduction increased somewhat in mid-winter and again in the spring, but no well-defined breeding season could be discerned. This is in contrast to studies which have been made in northern waters, where seasonal breeding in certain species is seen more clearly (Pierce, 1941; Clarke *et al.*, 1943).

Abundance. The numbers of *S. hispida* captured per cubic meter of water sieved are shown in Tables I, II, and III. There was a sizable increase in the population in May at all A stations and in June at 2A and 3A; however, there were

TABLE VI

Stages of maturity for S. hispida
Data are included from all stations
at which specimens were found

Date	Stage I		Stage II		Stage III		Total
	No. spec.	%	No. spec.	%	No. spec.	%	
1948							
Nov.	62	75	17	20	4	5	83
Dec.	31	72	12	28	0	0	43
1949							
Jan.	187	91	17	8	3	1	207
Feb.	168	84	29	14	3	2	200
Mar.	42	50	33	39	9	11	84
Apr.	79	54	67	45	1	1	147
May	155	80	38	19	2	1	195
June	141	49	139	48	9	3	289
July	99	54	65	35	19	11	188
Aug.	213	78	49	18	10	4	273
Sept.	370	74	116	23	16	3	502
Oct.	156	73	50	23	8	4	214
Nov.	54	83	10	15	1	2	65
Dec.	72	95	4	5	0	0	76
1950							
Jan.	33	73	12	27	0	0	45

still numbers of young Stage I forms present during the other months of the year (Table IV) which is consistent with the evidence that breeding is continuous the year round.

A comparison may be made of the abundance of *S. elegans* over Georges Bank, off Massachusetts, during five cruises made in 1940 (Clarke *et al.*, 1943) with the number of *S. hispida* taken at three stations on the west coast for corresponding months. The maximum concentration of *S. elegans* for each cruise has been selected for comparison.

TABLE VII

*Comparison of catches of S. elegans from Georges Bank, Massachusetts and S. hispida from the west coast of Florida**

	January	March	April	May	June
Georges Bank					
1940	26	5	11	43	112
West Coast of Florida					
1949					
Station 1A	2	41	78	197	23
Station 2A	15	33	12	45	46
Station 3A	13	46	52	223	481

* Figures represent individuals per cubic meter.

With some exceptions, the numbers of individuals are higher along the west coast than they are over Georges Bank. Of course, two different species are being compared; nonetheless, the data do suggest that population increases in northern waters are not always greater than population increases for similar species in the south during the breeding season.

Sagitta helenae Ritter-Zahony (Fig. 4)

Sagitta helenae Ritter-Zahony, 1910, p. 134, 1911a, pp. 18, 1911b, p. 20; Michael, 1919, p. 239; Thomson, 1947, p. 36; Moore, 1949, p. 26.

Description. This species was described by Ritter-Zahony (1910) from specimens taken near Dry Tortugas, Florida. It is a medium sized form with rather heavy body musculature and a large number of anterior teeth. The number and arrangement of these teeth allow it to be distinguished from the very similar though smaller *S. hispida* Conant. The anterior teeth in *S. helenae* are more abundant for any given size than are the teeth in *S. hispida.* In the 6–10 mm. range the difference is apt to be two to three anterior teeth; in the larger specimens

TABLE VIII

Measurements of S. helenae from the west coast of Florida

Length mm.	Tail segment %	Hooks	Anterior teeth	Posterior teeth
6–7	25–27	6–7	8–10	7–10
8–9	26–28	6–8	9–12	9–13
10–11	25–26	7–8	11–16	11–12
12–13	24	7	15	11–12
14–15	24–25	7–8	15–16	11–14

the difference is greater, usually amounting to 6–8 more teeth on each side. The anterior teeth in *S. helenae* exhibit a very noticeable overlap a short distance back from the points. In the majority, the teeth stand out from the head and resemble, in miniature, a small curved fan.

Distribution. This was the most common chaetognath in the plankton at station 1C, 2C and on occasions at 3C. A form widely ranging along the west coast, it was customarily found in water of 34–35$^0/_{00}$. It is significant that its presence at any station was with few exceptions assurance that *S. hispida* would be absent or found there in only minor numbers (Tables I, II and III). The Cedar Key area offered the least favorable environment for this species. It was never taken there at either 3A or 3B stations and in a number of instances it was absent from 3C as well.

Further information concerning the distribution of *S. helenae* along the west coast was obtained from a number of scattered samples taken over a period of several years. Two samples taken 30 and 50 miles southwest of Bradenton Beach, October, 1949, contained *S. helenae* in some abundance. A plankton tow made 12 miles northwest of Key West in July, 1946 contained a few *S. helenae.* Other samples taken in the close vicinity of Key West and in Florida Bay contained no representatives of this species. This information strengthens the evidence that *S. helenae*

will not tolerate indefinitely conditions where the salinity is several parts per thousand below normal, but will be found in high salinity water over the continental shelf along the west coast of Florida. No positive information concerning its offshore distribution in the Gulf is at present available.

Breeding. Evidence for year round breeding of *S. helenae* is seen in the abundance of Stage I individuals in every month. The general distribution of all stages during the year makes it difficult to select periods in which reproduction was accelerated. The large increase in the numbers of young individuals in May and June suggests that this was a time when breeding had increased.

TABLE IX

Stages of maturity for S. helenae
Data are included from all stations
at which specimens were found

Date	Stage I		Stage II		Stage III		Total
	No. spec.	%	No. spec.	%	No. spec.	%	
1948							
Nov.	181	90	17	9	2	1	200
Dec.	108	87	15	12	1	1	124
1949							
Jan.	231	94	16	6	0	0	247
Feb.	50	62	19	23	12	15	81
Mar.	243	90	23	9	3	1	269
Apr.	53	58	33	36	5	6	91
May	315	88	41	11	2	1	358
June	155	83	27	14	6	3	188
July	21	25	50	59	14	16	85
Aug.	87	94	6	6	0	0	93
Sept.	63	67	31	33	0	0	94
Oct.	29	71	7	17	5	12	41
Nov.	148	92	13	8	0	0	161
Dec.	29	88	4	12	0	0	33
1950							
Jan.	159	73	44	21	14	6	217

Abundance. The abundance of *S. helenae* at the C stations is generally similar to the number of *S. hispida* at the A stations (Tables I, II and III). The optimum location sampled regularly was 1C with 2C next. The largest number per cubic meter found at any one time was 325 on 18 June 1949 at 1C.

Sagitta tenuis Conant (Fig. 4)

Sagitta tenuis Conant, 1896, p. 213; Fowler, 1906, p. 61; Michael, 1911, p. 72, 1919, p. 239; Germain and Joubin, 1916, p. 51. (?); John, 1933, p. 4.
Sagitta friderici Ritter-Zahony, 1911a, p. 19, 1911b, p. 21.

Description. Described by Conant in 1896 from specimens collected June, 1893, Kingston Harbor, Jamaica. Several syntypes have been deposited by him in the

United State National Museum (Cat. No. 4986). A comparison of these specimens with the ones collected on the west coast revealed that they were the same species. Again there is an inadequate description and no figure. The following data were listed in the original description:

Maximum length	Tail segment	Hooks	Anterior teeth	Posterior teeth
5.25 mm.	25 per cent approx.	7–8	4–5	7–10

The greatest discrepancy in the above figures lies in the smaller number of posterior teeth listed by Conant. This is probably not very significant in view of the very small specimens with which he had to work.

These are small slender chaetognaths. The fins are completely set with rays with posterior and caudal fins touching the seminal vesicles in mature individuals. The anterior fins reach forward to the ventral ganglion. The corona is elongate, extending from beyond the eyes more than halfway to the ventral ganglion. The seminal vesicles are slender with a rounded knob on the anterior end when mature. The posterior fins lie more on the tail segment than on the trunk. A line drawn along the edges of each group of anterior teeth would meet medially at an acute to right angle. The collarette is small, evident at the junction of the head and body. The ovaries are short, not usually extending much beyond the posterior fin. The individual eggs are few and rather large in the short ovary. *S. tenuis* can readily be separated from *S. hispida* by the shape of the seminal vesicles and the arrangement of the anterior teeth.

Ritter-Zahony (1910, p. 141) attributes characters described by Conant for *S. tenuis* to *S. bipunctata* and fails to recognize it as a valid species. Michael (1911, p. 73) recognizes *S. tenuis* as a species in good standing. On the other hand, *S. friderici* Ritter-Zahony (1911b) is almost certainly a synonym of *S. tenuis*. The description and figures of *S. friderici* are in complete accord with the specimens from the west coast of Florida, and Conant's *S. tenuis*. Ritter-Zahony obtained his collection from the neighborhood of Porto Grande, Cape Verde Islands.

TABLE X

Measurements of S. tenuis from the west coast of Florida

Length mm.	Tail segment %	Hooks	Anterior teeth	Posterior teeth
6	25	8	6	15
7	26	7–8	6–7	18–19
8	27	8	7	15

Distribution. The most widely distributed chaetognath on the west coast, it was found on occasion at every station, although somewhat less abundantly at the A stations. It also occurs on the east coast and apparently in greater numbers than on the west coast. Specimens were obtained from Salerno, and St. Augustine, Florida, and Beaufort Inlet,[6] North Carolina.

[6] Beaufort Inlet material obtained through the courtesy of Dr. W. H. Sutcliffe, Jr., Duke Marine Laboratory, Beaufort, N. C.

Breeding. This species appears to breed to some extent throughout the year. Mature forms were found in every month, with the exception of October when very few specimens were captured in any stage.

Abundance. *S. tenuis,* more than either of the two previously discussed species, shows some seasonal occurrence. It was present in numbers during winter and spring but diminished or disapppeared during the summer and early fall months. This coincides with the period of highest water temperature (Fig. 2) and possibly

TABLE XI

Stages of maturity for S. tenuis
Data are included from all stations
at which specimens were found

Date	Stage I		Stage II		Stage III		Total no.
	No.	%	No.	%	No.	%	
1948							
Nov.	12	32	18	47	8	21	38
Dec.	60	58	32	31	12	11	104
1949							
Jan.	45	30	63	40	45	30	153
Feb.	20	43	14	30	13	27	47
Mar.	66	38	60	34	49	28	175
Apr.	39	35	53	47	21	18	113
May	0	0	25	58	18	42	43
June	26	51	21	41	4	8	51
July	1	4	16	64	8	32	25
Aug.	0	0	5	50	5	50	10
Sept.	2	8	15	62	7	30	24
Oct.	0	0	3	100	0	0	3
Nov.	7	33	11	52	3	15	21
Dec.	0	0	17	61	11	39	28
1950							
Jan.	3	7	15	35	25	58	43

the high water temperatures affected it adversely. Although widely distributed, it did not occur in as large numbers as did *S. hispida* and *S. helenae.*

Sagitta enflata Grassi (Fig. 4)

Sagitta enflata Grassi, 1881, p. 213; Beraneck, 1895, p. 153; Aida, 1897, p. 15; Doncaster, 1902, p. 210; Fowler, 1906, p. 8; Ritter-Zahony, 1910, p. 139, 1911a, p. 13, 1911b, p. 16; Michael, 1911, p. 28, 1919, p. 242; Bigelow, 1926, p. 334; Burfield and Harvey, 1926, p. 111; John, 1933, p. 1; Tokioka, 1939, p. 126, 1940a, p. 2, 1940b, p. 369, 1942, p. 527; Redfield and Beale, 1940, p. 472; Clarke, Pierce and Bumpus, 1943, p. 221; Thomson, 1948, p. 18, 1947, p. 11; Moore, 1949, p. 25; Davis, 1949, p. 88; King, 1949, p. 119.
Sagitta flaccida Conant, 1896, p. 213; Doncaster, 1902, p. 211.
Sagitta gardineri Doncaster, 1902, p. 212; John, 1933, p. 2.
Sagitta inflata Germain and Joubin, 1916, p. 33.

Description. *S. enflata* is a medium sized, well known and easily recognized chaetognath. The overlapping anterior teeth, the short corona situated on the head, the small knob-like seminal vesicles, together with the flaccid body are features which characterize this species.

Distribution. Along the west coast *S. enflata* occurred seldom or never at the A and B stations and was present in reduced numbers and then only at scattered intervals at 3C (Tables I, II and III). It occurred normally in the samples in which *S. helenae* was found, and if *S. helenae* were absent *S. enflata* was usually absent as well. As was the case for *S. tenuis*, it almost completely disappeared during late summer and early fall. *S. enflata* appears to have little tolerance for either low salinities or persistent high temperatures. In the tropics the ability of *S. enflata* to perform vertical migrations might remove it when necessary from the very warm

TABLE XII

The measurements of S. enflata from the west coast of Florida

Length mm.	Tail segment %	Hooks	Anterior teeth	Posterior teeth
8–9	15–18	8–9	5–6	8
10–11	17	8–9	5	8
12–13	16–18	8–9	6–7	11
14–15	16–17	8–9	7–9	10–13
16–17	17	8	9	14
18–19	15–17	8	7–9	13–14
20–21	15	9	11	15

surface waters. Moore (1949) states that in the Bermuda area its mean day level was 115 meters.

S. enflata is typically an offshore species. It has a cosmopolitan distribution in all the warmer seas. Ritter-Zahony (1911b) states that its range is between 40°N and 40°S latitude. These limits appear to be generally true as far as the existence of a permanent population is concerned, although there are records of its occurrence farther north (Redfield and Beale, 1940).

Breeding. The data are too scattered to permit valid conclusions concerning the breeding season; however, the presence of Stage I forms at all seasons suggests that the period of reproduction is a long one in this area.

Abundance. Judged on a year round basis this species was fourth in abundance in the west coast area (Tables I, II and III). Usually occurring in numbers of less than 50 per cubic meter, it occasionally exceeded this figure in the winter months.

Krohnitta pacifica (Aida) (Fig. 4)

Krohnitta pacifica Tokioka, 1939, p. 135, 1940a, p. 7, 1942, p. 546; Thomson, p. 22.
Krohnia pacifica Aida, 1897, p. 19; Doncaster, 1902, p. 215; Fowler, 1906, p. 24.
Krohnitta subtilis (partim) Ritter-Zahony, 1910, p. 140, 1911a, p. 44, 1911b, p. 32.
Eukrohnia pacifica Michael, 1911, p. 76.

Description. Ritter-Zahony (1910) established the genus *Krohnitta* and synonymized *K. pacific* under *K. subtilis*. As indicated by Tokioka (1939) and

TABLE XIII

Stages of maturity for S. enflata
Data are included from all stations
at which specimens were found

Date	Stage I		Stage II		Stage III		Total no.
	No.	%	No.	%	No.	%	
1948							
Nov.	20	76	3	12	3	12	26
Dec.	79	79	16	16	5	5	100
1949							
Jan.	119	75	20	12	20	12	159
Feb.	115	89	11	9	3	3	129
Mar.	120	74	36	22	7	4	163
Apr.	16	53	5	17	9	30	30
May	81	88	11	12	0	0	92
June	10	77	3	23	0	0	13
July	1	100	0	0	0	0	1
Aug.	2	100	0	0	0	0	2
Sept.	4	100	0	0	0	0	4
Oct.	1	50	1	50	0	0	2
Nov.	82	99	0	0	1	1	83
Dec.	42	93	3	7	0	0	45
1950							
Jan.	42	71	17	29	0	0	59

Thomson (1947) these are separate and clearly defined species. *K. pacifica* is a shorter, broader form with a relatively large ovary extending beyond the border of the posterior fin when mature. The ovary in *K. subtilis* is small, hardly as long as the width of the body.

Distribution. The occurrence of *K. pacifica* along the west coast was sporadic. It was found in some of the fall and winter samples at 1B, 1C and 2B, 2C in small numbers (Tables I, II and III). Only two specimens were taken in the quantita-

TABLE XIV

Measurements of K. pacifica from the west coast of Florida

Length mm.	Tail segment %	Hooks	Teeth
5	28–29	9	11
6	27–28	8–9	12
7	30	9	12

tive sampler in the Cedar Key area. This latter region is clearly the least favorable of the three for this species.

Breeding. Not enough specimens were collected to warrant definite conclusions on the breeding of this species; however, whenever any number were taken, some of them were almost invariably mature. This strongly suggests that the breeding season for *K. pacifica* is a long one in this region. Stage I individuals

were rarely recorded because their minute size (two–three mm.) made selection from the samples difficult and subject to error.

Abundance. It was clearly the least abundant of the chaetognaths collected. The maximum number encountered was 28 per cubic meter in November, 1949 at station 1B. Although it did not appear simultaneously at the Ft. Myers Beach or

TABLE XV

Stages of maturity for K. pacifica
Data are included from all stations
at which specimens were found

Date	Stage I	Stage II	Stage III	Total
	No.	No.	No.	
1948				
Nov.	0	14	44	58
Dec.	0	8	3	11
1949				
Jan.	0	2	2	4
Feb.	0	0	4	4
Mar.	0	1	2	3
Apr.	2	3	3	8
May	0	0	0	0
June	0	0	0	0
July	0	1	0	1
Aug.	0	1	1	2
Sept.	0	4	3	7
Oct.	0	4	12	16
Nov.	1	8	6	15
Dec.	0	1	8	9
1950				
Jan.	0	7	8	15

Bradenton Beach stations, it was captured on 13 occasions in each area and in roughly comparable numbers.

Regeneration in Chaetognaths

An unusual phenomenon was observed in a number of specimens of *S. helenae* and *S. enflata* (26 were taken from the samples). These individuals were found to have lost their heads, together with a short section of the trunk and were in various stages of head regeneration (Fig. 5). This condition was at first attributed to accidental damage encountered in the net; however, as additional specimens were encountered in which various degrees of head regeneration could be observed, it was concluded that this was not an unusual occurrence and that the loss of this section of the body did not necessarily kill the individual. No instances of the re-growth of the tail or posterior end of the body were observed.

Upon the loss of the head the trunk tissues contract tightly together at the severed end. This gives the appearance of the tied end of a sausage casing. The

head begins to form within the constricted end. The eyes appear early, followed by the mouth and finally jaws appear.

Other features which lend interest to this observation are that although many hundreds of *S. setosa* and *S. elegans* from northern latitudes were carefully inspected in other studies, no evidence of regeneration of the head was ever observed. No mention of such phenomena has been recorded in the literature reviewed.

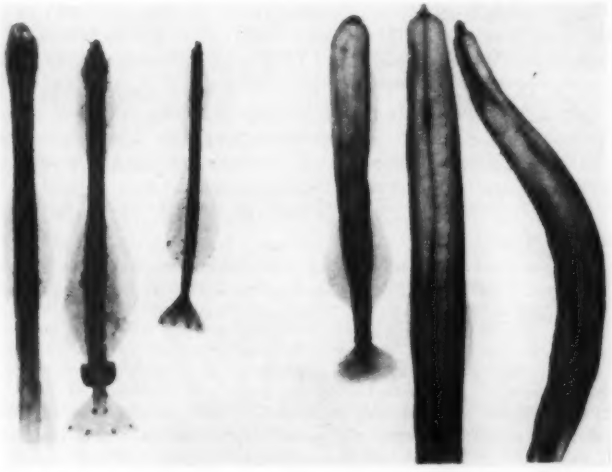

FIGURE 5. Chaetognaths showing various stages of head regeneration. The three specimens on the left are *S. helenae*. The three on the right are *S. enflata*.

DISCUSSION

A comparison of the three base areas shows a considerable similarity between Bases 1 and 2 from the point of view of occurrence and abundance of the five species. The Cedar Key area, with its reduced salinity and the predominance of *S. hispida,* showed somewhat greater differences in its chaetognath fauna from the other two.

The distribution of the chaetognaths along the coast is consistent with the hydrographic conditions operating there. Inshore, there is a very appreciable drift of water northward along the coast (United States Hydrographic Office, Current Charts, Central American Waters). Although the figures vary considerably from month to month, a rough average of 6–8 miles per day would indicate the magnitude of the movement involved. In the Bradenton Beach area this northward counter current may at times exceed 20 miles per day. The fact that the movement of water is parallel to the coast, together with the relatively mild tides in this area, allows salinity and faunal areas paralleling the coast to persist almost indefinitely. At the same time, this northward movement of water is responsible for the wide distribution of the chaetognaths within the respective areas of salinity. It was significant to find the difference in distribution between *S. hispida* on the one hand and *S.*

helenae and *S. enflata* on the other. Along the west coast of Florida the former was characteristically found in the inshore water of low salinity, whereas the two latter species were seldom taken in company with *S. hispida*. The year-round persistence of the difference in the distribution of the species substantiates the lack of lateral mixing which was also indicated by the salinity gradient found along the coast. There is reason to believe that these species might be successfully used as indication of the movement of water from offshore inward or from the inshore outward.

During the period of this study, despite a hurricane which traversed almost the length of the state on August 26–27, 1949 (Latour and Bunting 1949), no unusually high salinity records were obtained at the inshore stations, nor was there any evidence from chaetognath distribution that species occurring offshore had been swept inshore in any numbers. In fact, the reverse appears to have taken place. On 12 September, 16 days following the storm, *S. hispida* was taken at station 1C which was the first time in many months that it had appeared at this station, and *S. helenae* was absent for the first time. *S. hispida* was taken at 2C for the first time the following month.

Additional collections of chaetognaths are needed from points far out into the Gulf and at various depths to allow us to define better the offshore range of such species as *S. helenae, S. tenuis* and *K. subtilis*.

Summary

1. From November, 1948 through January, 1950 quantitative plankton collections were made at monthly intervals from nine stations along the west coast of Florida.

2. Surface water temperatures and salinities were taken at each station. The warmest water temperatures were similar at all stations but the coldest water by several degrees was found at the Cedar Key station (3A).

3. The inshore water at all stations showed reduced salinity. This was correlated with rainfall. The salinity increased from the inshore to the offshore stations.

4. Three maturity stages were described for the chaetognaths based on development of testes and ovaries.

5. The chaetognaths collected were: *S. hispida, S. helenae, S. tenuis, S. enflata, K. pacifica*. One of these, *S. hispida*, exists characteristically in dilute inshore water. *S. helenae* and *S. enflata* were normally absent at the inshore stations where *S. hispida* was abundant. They were most numerous at the C stations where the salinity of the water varied around $35^0/_{00}$. *S. tenuis* appeared to be rather tolerant of fluctuations in salinity although in the Cedar Key area, where the lowest salinities were encountered, it was noticeably scarce at the inshore stations. The catches of *K. pacifica* were too few and scattered to give a good picture of its local distribution. It was more abundant in waters of high salinity than elsewhere.

6. The data indicated that breeding was, with some fluctuations, continuous the year round.

7. *S. hispida* and *S. helenae* were found at all seasons. Increased numbers of Stage I forms appeared in the late spring and summer, resulting in an over-all increase in the population. *S. tenuis* and *S. enflata* were noticeably most abundant during the colder months.

8. A northward movement of the coastal water helps distribute the chaetognaths and apparently does not interfere seriously with the persistence of water of reduced salinity in the inshore areas.

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SOME ASPECTS OF RESPIRATORY METABOLISM DURING METAMORPHOSIS OF NORMAL AND DDT-RESISTANT HOUSE FLIES, MUSCA DOMESTICA L.

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In this study, the respiratory metabolism of two strains of house fly, one of them resistant to DDT, has been investigated in order to ascertain some of the biochemical events associated with developmental processes, and to contribute to our scant knowledge of the mechanism of insect resistance to insecticides.

Inasmuch as both DDT and cyanide are known to inhibit cytochrome oxidase, it seemed worthwhile to compare the effects of cyanide on normal and resistant strains of a single species. Pupae were used in these experiments because measurements of their oxygen consumption could be made without the interference of bodily activity, and also because they provide an opportunity for gaining a better understanding of biochemical events during metamorphosis.

General reviews of the respiratory metabolism of insects during this stage of development have been compiled by Needham (1942) and Wigglesworth (1947). The inhibitory action of cyanide, azide and carbon monoxide on cytochrome oxidase has been studied by Keilin and Hartree (1939) and by others. With insect material, the effects of one or more of these inhibitors have been reported for eggs of the grasshopper, *Melanoplus differentialis* (Bodine and Boell, 1934); for larvae of the codling moth, *Cydia pomenella* (Graham, 1946); and for pupae of *Drosophila melanogaster* (Wolsky, 1938). Collier (1940) and Zukel (1944) studied the inhibition of cytochrome oxidase by phenothiazine, while Sacktor (1949) and Johnston (1950) found that DDT also inhibits this enzyme.

The development of insect resistance to insecticides has become a problem of major economic and, potentially, medical importance. Quayle (1916) and others showed that certain scales had become resistant to cyanide. Cyanide resistance has been reported also for *Drosophila melanogaster* and an aphid, *Aphis gossypii* (Boyce, 1928), as well as for the confused flour beetle, *Tribolium confusum* (Gough, 1939). Screw-worms, *Callitroga americana*, resistant to phenothiazine were found by Knipling (1942). In recent years, reports of house flies and other insects resistant to DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) and other synthetic insecticides have become commonplace. The subject has been reviewed by Babers (1949). Since then, experiments by Sternburg and Kearns (1950) and Sternburg, Kearns and Bruce (1950) revealed that all stages of several DDT-resistant strains of house fly were capable of converting DDT to non-toxic DDE (1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene), whereas only pupae of a normal strain could metabolize DDT to DDE. In partial agreement with the above results, Perry and Hoskins (1950) found that adults of both a resistant and normal strain could detoxify DDT but that the extent of this detoxification was greater with the resistant strain. Despite these observations, it may still be said that

little is known about the physiological mechanisms concerned in resistance to insecticides, since the mode of action of the compounds themselves is seldom understood.

Methods

The DDT-resistant strain of house flies was derived from the Ellenville line. Details concerning this stock and the rearing procedure have been reported elsewhere (Sacktor, 1951).

Warburg manometers were used for determinations of normal and cyanide inhibited oxygen consumption. Four pupae of a given age were employed per determination. In measuring the normal oxygen consumption the pupae were placed in the main compartment of the flask, the center well containing 0.2 ml. of 0.5 *M* KOH. To determine the consumption during cyanide inhibition, the pupae were placed in the side arm of the vessel. As recommended by Robbie (1946), the center well contained KCN-KOH or $Ca(CN)_2$-$Ca(OH)_2$ mixtures, depending on the experimental concentration of cyanide used. The former mixture was used for determinations of the inhibition with 3×10^{-4} *M* cyanide, the latter with 4×10^{-3} and 5×10^{-2} *M*. When measuring the inhibition with the lowest cyanide concentration, the main compartment of the flask contained 1.0 ml. of *M*/150 phosphate buffer, pH 7.0, and 3×10^{-4} *M* KCN. Nothing was placed in the main compartment when determining the inhibition with higher cyanide concentrations.

The $Ca(CN)_2$-$Ca(OH)_2$ mixtures were prepared according to the procedure of Robbie and Leinfelder (1945). The HCN concentration of these mixtures was determined by the method of Liebig (1851). The experimental HCN concentrations were interpolated or extrapolated from the data given by Robbie (1946).

The flasks were allowed to equilibrate for 30 minutes at 30° C. with a shaking rate of 120 per minute. The oxygen uptake was thereafter recorded every 10 minutes for one hour.

Cytochrome oxidase activity was measured in a Beckman spectrophotometer at 5500 Å. The procedure described in detail in a previous paper (Sacktor, 1951) was followed with but one minor modification: For each determination 5 pupae were homogenized in 5.0 ml. iced distilled water. A 1.0 ml. aliquot of this stock homogenate was diluted five-fold, and paired determinations were made using 0.5 ml. of the diluted homogenate.

To determine the mortality of pupae exposed to cyanide vapor, the pupae were collected from the Warburg flasks immediately after respiration studies. These pupae were then washed, dried and placed in petri dishes. The non-emergence of the flies was used as the death criterion.

Results

The effect of cyanide at various concentrations on the oxygen consumption of normal pupae is shown in Figure 1. Each point on the curves representing the normal oxygen consumption and that of pupae exposed to 0.3×10^{-4} *M* cyanide indicates the average of 12 determinations. The remaining points represent averages of from 4 to 12 determinations each. The per cent of respiration insensitive to cyanide varied with the age of the pupae, as recorded in Table I.

TABLE I

Per cent of pupal respiration insensitive to cyanide

Age days	Normal strain			Resistant strain		
	M conc. of HCN					
	3×10^{-4}	4×10^{-3}	5×10^{-3}	3×10^{-4}	4×10^{-3}	5×10^{-3}
0	16	8	6	19	14	8
1	38	36	21	72	69	61
2	45	39	32	67	46	68
3	73	31	10	87	61	37
4	83	24	15	65	25	18

Similar data for the DDT-resistant strain are given in Figure 2 and Table I.

Figure 3 shows the change in cytochrome oxidase activity in both strains of pupae in relation to their development. Each point represents the mean and its standard error for 14 to 26 determinations.

In Table II is shown for both strains of pupae the mortality due to exposure to different cyanide concentrations. These results were obtained by combining those pupae, 16 to 48 in number, used under each experimental condition.

TABLE II

Per cent mortality of pupae exposed to cyanide vapor

Age days	Normal strain			Resistant strain		
	M conc. of HCN					
	3×10^{-4}	4×10^{-3}	5×10^{-3}	3×10^{-4}	4×10^{-3}	5×10^{-3}
0	94	100	100	95	100	100
1	25	92	100	9	69	100
2	36	100	100	22	100	100
3	75	100	100	22	88	100
4	88	100	100	29	96	100

DISCUSSION

Oxygen consumption

The oxygen consumption of normal and resistant house fly pupae exhibited the characteristic U-shaped curve during their development. These results are in agreement with a multitude of other investigations with holometabolic insects (Haub and Hitchcock, 1941, with Phormia; Frew, 1929, with Calliphora; Wolsky, 1938, with *Drosophila melanogaster;* Dobzhansky and Poulson, 1935, with *D. pseudoobscura;* Ludwig, 1931, with Popillia; Lindgrem, 1935, with Tribolium; and others).

Figures 1 and 2 reveal that the oxygen consumption of the white pre-pupae (zero age) was considerably higher then than it was immediately prior to the

emergence of the adults. This is an apparent disagreement with some other investigations. Oxygen consumption of the early stages, however, was measured in these experiments within one-half hour after puparium formation. This is as much as 10 hours earlier than has been reported by some other investigators. It is

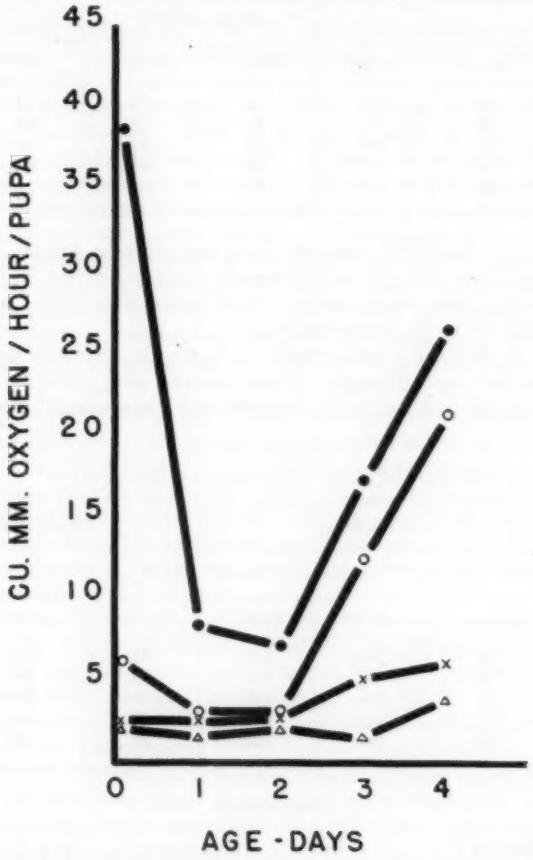

FIGURE 1. The effect of cyanide at various concentrations on the oxygen consumption of normal house fly pupae. Solid circles represent normal oxygen consumption. Open circles, crosses and triangles represent oxygen consumption in presence of, respectively: 3×10^{-4} *M*, 4×10^{-3} *M*, and 5×10^{-2} *M*, cyanide.

thus evident that oxygen consumption must decrease rapidly at this critical period. This interpretation is in agreement with measurements of oxygen consumption in *Drosophila pseudoobscura* by Dobzhansky and Poulson (1935), and with the unpublished data of Bodenstein and Sacktor on larval and pupal cytochrome oxidase in *D. virilis*, and of Levenbook (1950) on respiration in Calliphora.

The significance of the U-shaped curve is not fully understood. Gaarder (1918) has shown that the fall in oxygen uptake is not due to a reduction in the oxygen tension in the tissues. As pointed out by Needham (1942), the idea "that it (the change in oxygen uptake) represents the metabolic consequences of

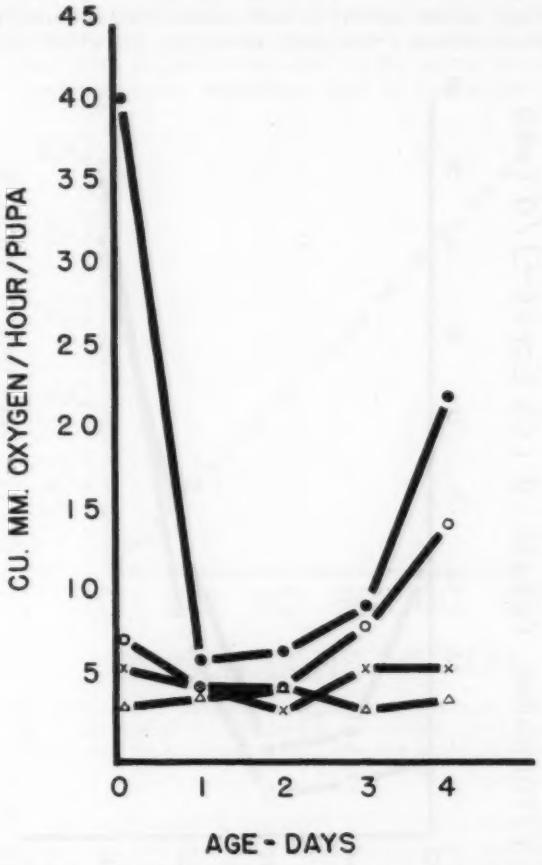

FIGURE 2. The effect of cyanide at various concentrations on the oxygen consumption of DDT-resistant house fly pupae. Solid circles represent normal oxygen consumption. Open circles, crosses and triangles represent oxygen consumption in presence of, respectively: 3×10^{-4} *M*, 4×10^{-3} *M*, and 5×10^{-2} *M* cyanide.

complete histolysis followed by new tissue differentiation . . . cannot be true." Such a view, which was once widely accepted, is not consistent with the histological data found by Dobzhansky and Poulson (1935) for Drosophila pupae. Agrell (1947) assumed that the U-shaped course of respiration is, to a certain degree, connected with variations in the activity of the dehydrogenase systems. Wolsky

(1938) attributed the changes in oxygen consumption to the quantity or activity of the cytochrome system. The results of the present study support Wolsky's interpretation in part, but point also to additional complicating factors.

Cytochrome oxidase

The cytochrome oxidase activity of both strains changes during pupal development. The activity follows a U-shaped curve (Fig. 3), which has, in general, a

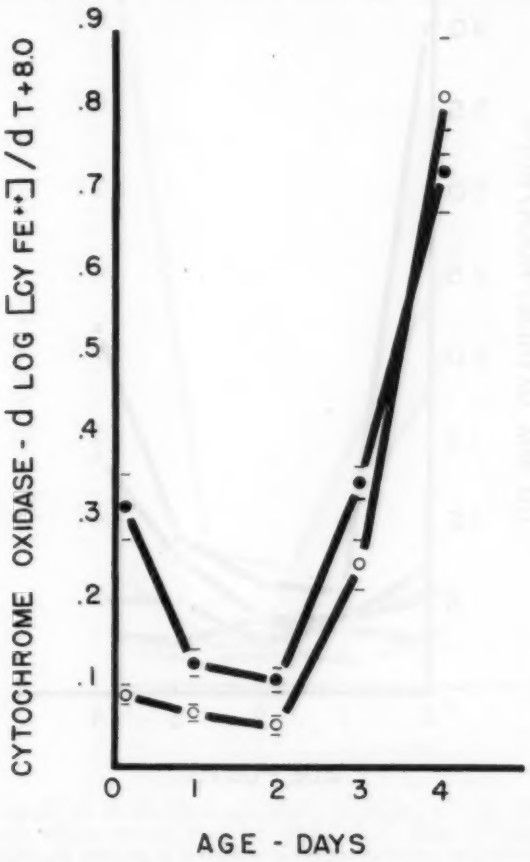

FIGURE 3. The change in cytochrome oxidase activity in both strains of house fly in relation to their pupal development. Solid circles represent normal strain; open circles represent DDT-resistant strain.

similar appearance to that of oxygen consumption *in vivo*. The relationship of respiration to cytochrome oxidase activity is shown in Figure 4 (Boell, 1945, found a like comparison with developing embryos of *Amblystoma punctatum*).

The broken line drawn through the origin is based on the assumption of 1 : 1 correspondence between oxygen uptake and cytochrome oxidase activity. In plotting these data, the total measured oxygen consumption was corrected by subtracting the average cyanide insensitive respiration of 2.0 cu. mm./hr./pupa for the normal strain and 4.0 cu. mm./hr./pupa for the resistant strain. The graph shows that an approximately linear relationship exists between cyanide sensitive respiration and cytochrome oxidase activity during pupal development for 1–4 days of age.

It will be noted from Figure 4 that some of the points reveal that there is slightly more cyanide sensitive respiration than is accountable by cytochrome

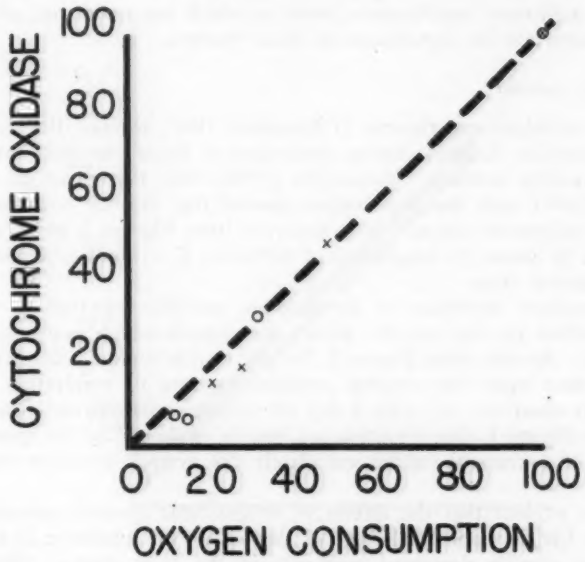

FIGURE 4. The relationship of oxygen consumption to cytochrome oxidase activity. All numbers in per cent of the highest O_2 consumption and oxidase activity observed. Crosses represent normal strain; open circles represent DDT-resistant strain. For further details see text.

oxidase activity. An examination of Figures 1 and 2 shows that these points correspond to pupae one and two days old. Further, the data obtained for zero age pupae are not included in the curve of Figure 4. These data (compare Figs. 1 and 3) indicate that there is at this stage a great excess of cyanide sensitive respiration not accounted for by cytochrome oxidase, and thus suggest that other cyanide sensitive systems are contributing to the total oxygen consumption. Such systems might involve tyrosinase or catalase, which are cyanide sensitive enzymes likely to be present in animal tissues. But from the existing evidence (Fink, 1930; Williams, 1936) there seems to be little correlation between catalase content of insects and the intensity of their respiration. Dennell (1947), however, related tyrosinase activity to puparium formation. In his review of tyrosinase in insects,

Sussman (1949) reported that tyrosinase activity increased until pupation, whereupon there was a rapid decrease.

Thus, the present experiments provide some evidence partially substantiating Wolsky's hypothesis, that changes in oxygen consumption during metamorphosis are related to the activity of cytochrome oxidase. But there are other factors here, in addition to the cytochrome system, which contribute to the total respiration. These are a cyanide insensitive system (which will be discussed later) and another cyanide sensitive system, perhaps tyrosinase. The latter is significant mainly in the early stages of pupal development.

The factors that cause changes in the cytochrome oxidase activity are not known, and additional experiments, some of which are underway, are necessary in order to ascertain the significance of these changes.

Inhibition by cyanide

Cyanide inhibition experiments (Commoner, 1940; Robbie, 1949; and others) reveal considerable variation among organisms in regard to their cyanide sensitive and insensitive systems. Runnström (1930) with the sea urchin and Bodine and Boell (1934) with the grasshopper showed that cyanide had various effects on different embryonic stages. It is apparent from Figures 1 and 2 and Table I that in house fly pupae the magnitude of inhibition by cyanide is dependent on the the developmental stage.

Upon complete inhibition of the cyanide sensitive respiration, the cyanide stable respiration (in cu. mm./hr./pupa) was approximately constant throughout development. As seen from Figure 5, the per cent inhibition was therefore found to be dependent upon the original cyanide-free rate of respiration. Only one deviation was observed, *i.e.*, with 4 day old pupae of the normal strain. As can be seen from Figure 1, this deviation can best be explained by the apparent failure of 5×10^{-2} *M* cyanide to inhibit completely the cyanide sensitive respiration of that stage.

It is also evident that the effects of submaximal cyanide concentrations depended upon the developmental stage of the pupae, for inhibition at a given concentration of cyanide decreased markedly in the later stages. Similar results were obtained by Wolsky (1938), with carbon monoxide and Drosophila pupae. In general terms, he proposed two explanations, namely; "(1) there may be changes in the physical-chemical properties of the medium in which the Warburg-Keilin system is reacting, affecting the velocity constants of the reactions or the solubility of the gases; (2) there may be a qualitative change in the Warburg-Keilin system, which alters the velocity constants of its reactions, so that it reacts more readily with O_2 and less readily with CO." He also noted that the work of Szorenyi and Tschepinoga (1936), who reported that in trained muscles the oxygen consumption increased and at the same time the respiration became more resistant to cyanide.

This partial inhibition by cyanide and carbon monoxide is of considerable interest and may reveal certain properties of the Warburg-Keilin system. Unfortunately the possible explanations are based partly upon conjecture, but a discussion of them may be worthwhile in directing future experimentation. Cook *et al.* (1931) and Cook and Haldane (1931) showed that with bacteria, at a given level

of cyanide or carbon monoxide, the sensitivity of the respiration was dependent on the nature of the substrate. They observed that the affinity constant (K = affinity of oxidase for O_2/affinity of oxidase for CO) with glucose was approximately 8 ×, 4 × and 2 × that when formate, succinate or lactate, respectively, were used. A similar effect was found by Ogston and Green (1935) for the respiration of yeast in various substrates. At a given cyanide level, the greatest oxygen consumption was with glucose as the substrate. The consumption decreased, in this order, when glycerophosphate, lactate, hexosediphosphate and hexosemonophosphate were used. There is, therefore, evidence that the cyanide

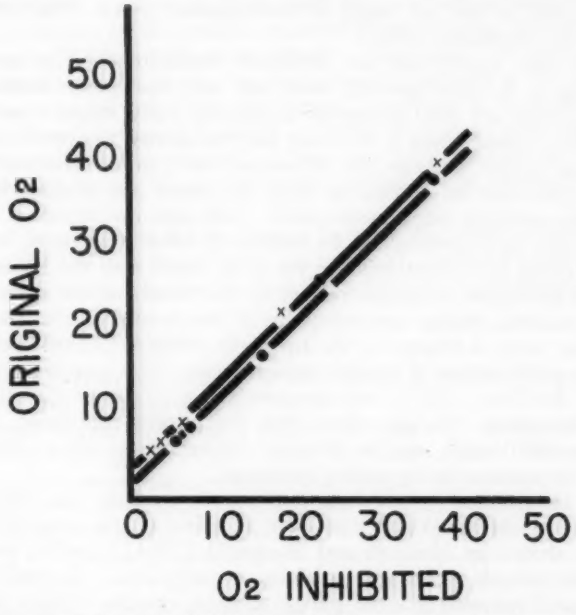

FIGURE 5. Amount of oxygen inhibited as a function of the original oxygen consumption. Ordinate, normal O_2 consumption in cu. mm./hr./pupa. Abscissa, amount of O_2 in cu. mm./hr./pupa inhibited by cyanide. Crosses represent DDT-resistant strain; closed circles represent normal strain. For further explanation see text.

(or carbon monoxide) sensitivity of the oxidation of various substrates is a function of the relative capacity of their specific dehydrogenases. Some dehydrogenases appear to require a greater degree of activity on the part of the oxidase, thus rendering the respiratory rate more sensitive to the decrease in oxidase activity by inhibitors.

During metamorphosis the developing insect utilizes reserve oxidizable substances. In many cases glycogen appears to be utilized throughout metamorphosis (for more details see Rockstein, 1950). As shown by Ludwig and Rothstein (1949) in the Japanese beetle, *Popillia japonica*, glycogen decreases most rapidly

during the first days of pupal life. Glucose also may serve as a substrate during the pupal stage. Evans (1932) found that the glucose content of the blowfly, *Lucilia sericata,* falls in young pupae but remains fairly constant during the later stages. A continuous utilization of glucose during metamorphosis occurred in the mealworm, *Tenebrio molitor,* (Evans, 1934). Courtois-Drilhon (1931), Crescitelli and Taylor (1935) and Ludwig and Rothstein (1949), however, have shown an increase in glucose during the prepupal and pupal period in several species of Lepidoptera and the Japanese beetle. This increase in glucose content is attributed to its conversion from glycogen. There are considerable differences in the utilization of fat during metamorphosis of insects. In general, fats seem to be used primarily in the late stages of development (Frew, 1929; Evans, 1934; and Ludwig and Rothstein, 1949).

The above data reveal that the substrates metabolized during metamorphosis vary according to the developmental stage and with the species studied. In general, carbohydrates are used primarily during the early stages whereas fats are consumed later. Since there is evidence that sensitivity to cyanide is dependent upon the nature of the substrate, the differences found in the effects of submaximal cyanide concentrations on developing house fly pupae may perhaps be related to changes in the substrate being metabolized. Although this inference is supported by analogy with facts determined in studies of other organisms, investigations suitable for testing this hypothesis are yet to be made with the house fly.

Another explanation, although related to the above, of the differences in effects of submaximal cyanide concentrations is the possibility that partial cyanide inhibition may cause a change in the hydrogen donators. Such alterations may even result in a stimulation of oxygen consumption. This possibility is excellently discussed by McElroy (1947) and for details it is recommended that reference be made to his paper. He has shown that the oxygen consuming reactions exhibited by normal tissues may be entirely different from those which maintain the residual respiration during partial inhibition.

Cyanide, in addition to inhibiting cytochrome oxidase, may also react with other metabolic participants and thus have an effect on the oxygen consumption *in vivo.* As shown by Marshall and Rosenfeld (1934), cyanide combines with aldehydic and ketonic substrates to form cyanohydrins. Recently, Lehninger (1950) reviewed the role of metal ions in enzyme systems. Since several heavy metals may react with cyanide, various enzymatic processes in the total respiratory metabolism may be altered. These alternatives should be kept in mind in considering the significance of the present results.

Cyanide insensitive respiration

Upon apparently complete inhibition of the cyanide sensitive respiration, there still remained a residual oxygen consumption. It is evident from Figures 1 and 2 that the cyanide insensitive respiration is relatively constant throughout metamorphosis, although different for each strain. In the normal strain the average cyanide insensitive oxygen consumption was 2.0 cu. mm./hr./pupa, whereas for the resistant strain it was twice as great. In Figure 5, by plotting original oxygen consumption against cyanide inhibited respiration, a line is obtained whose intercept on the ordinate represents the true value of the cyanide stable respiration.

The experimental values (Figs. 1 and 2) agree with the value obtained by such treatment of the data.

Although there are several enzymes, including cytochrome *b*, which are capable of reacting with oxygen and are not inhibited by cyanide, only for one of these, the "yellow enzyme" of Warburg and Christian (1932), are there sufficient data to estimate its significance in relation to the total respiration. The substrates undergoing oxidation through this enzyme are the hexosephosphates, citrate, glucose, and, to some extent, malate. Gourevitch (1937) reported a definite relationship in mammalian tissues between the quantity of flavin and the amount of cyanide insensitive respiration. Groen and Schuyl (1938) fed rats a flavin-free diet and found that the loss of flavin from the liver was accompanied by a corresponding reduction in the cyanide insensitive respiration. Normal and treated kidney, however, contained the same amount of flavin and the cyanide stable oxygen consumption was the same. Further evidence is found in the experiments of Pett (1936), who showed that when yeast is cultured in a medium containing cyanide, its flavin content is doubled and that correspondingly the cyanide insensitive respiration is also doubled. Thus, it is indicated that the absolute value of the cyanide stable respiration is directly related to the flavoprotein content of the tissue.

Although, as yet, flavin determinations have not been made on house fly pupae, the fact that the DDT-resistant strain has a greater cyanide insensitive respiration than the normal strain suggests the desirability of such a comparative study.

Comparison of the two strains.

The normal oxygen consumption of both strains of pupae during metamorphosis was approximately the same (Figs. 1 and 2).

It has been shown in Figure 3 that in all stages of development, except immediately before emergence of the adults, the pupae of the DDT-resistant strain have less cytochrome oxidase than the normal strain. At zero age the normal strain had approximately 3.5 times as much oxidase. Pupae one, two and three days old had 2.2 times, 2.4 times and 1.4 times as much oxidase, respectively. This suggests that in the resistant pupae a larger portion of the normal oxygen consumption passes through other respiratory systems, as is in fact demonstrated by the data given in Figures 1, 2 and 5, which reveal that the DDT-resistant pupae have twice the cyanide insensitive respiration of the normal strain.

As shown in Table I, oxygen consumption was inhibited by a given cyanide concentration to a greater extent in the normal strain than in the resistant strain. These results may be related to the finding that, as shown in Table II, the DDT-resistant pupae exhibit resistance to this poison.

The data in Table II also reveal that the lethal effect of a given cyanide concentration is dependent on the stage of development. In the normal strain, pupae one and two days old are least susceptible. This is in agreement with the fact that a larger portion of the normal oxygen consumption of these pupae is through the cyanide insensitive system. Of particular interest is the observation that, at a cyanide concentration of 3×10^{-4} *M*, 3 and 4 day old normal strain pupae mostly succumbed, whereas DDT-resistant pupae of the same age mostly survived. At this cyanide concentration the extent of inhibition in both strains was of the same

small magnitude. The possible mechanisms of this difference in survival of the two strains at these ages are speculative. They may be due to: (1) the dependence upon the cytochrome oxidase system for completion of certain developmental processes (Wolsky, 1937) which are stopped by the partial inhibition of the enzyme in the normal strain, whereas in the resistant strain, although the oxidase is also partially inhibited, these processes can proceed by the utilization of the flavin system; (2) more readily reversible inhibition of cytochrome oxidase in the resistant strain; (3) detoxification of cyanide by the resistant strain after dissociation of the inhibitor-enzyme complex. At this moment, we have no means of deciding which of these possible mechanisms, or combination of them, is actually employed.

It should also be noted that the mechanism of cyanide resistance in pupae may be different from that of DDT-resistance in adults. It was shown by Sacktor (1951) that DDT-resistant adults had approximately 50 per cent more oxidase than the normal strain. This suggests that one of the explanations for the resistance of adult house flies to DDT may be the greater oxidase activity, which will permit the continuance of essential physiological functions despite partial inhibition. In contrast, pupae may utilize a cyanide insensitive system for continuance of these functions. Further, the results of Sternburg and Kearns (1950), Sternburg, Kearns and Bruce (1950) and Perry and Hoskins (1950) in regard to the degradation of DDT by resistant strains must be considered. At present the mechanism of detoxification, or the possible role of DDE in preventing lethal effects of DDT is not known, and it is even possible that cytochrome oxidase is concerned in these mechanisms.

The present study suggests that DDT-resistance may be correlated with ability to maintain respiration in the face of partial inhibition of cytochrome oxidase. At the same time, the results draw attention to the complexity of the mechanisms concerned. These will require further investigation from the several other points of view noted above before a satisfactory interpretation can be reached.

Summary

1. Oxygen consumption follows a U-shaped curve during metamorphosis of normal and DDT-resistant strains of house flies. It is of the same order of magnitude in both strains.

2. Cytochrome oxidase activity during metamorphosis also follows a U-shaped curve. There is evidence that changes in oxygen consumption during this process are, in some respects, related to the activity of the oxidase.

3. A cyanide insensitive system, possibly flavin, and another cyanide sensitive system, probably tyrosinase, contribute to the total oxygen consumption. The latter system apparently contributes mainly during the early stages of metamorphosis.

4. The cyanide insensitive respiration of both strains remains relatively constant throughout development. The resistant strain has twice the cyanide insensitive respiration of the normal strain.

5. The DDT-resistant pupae have less cytochrome oxidase activity than normal pupae at all stages except immediately prior to emergence of the adult.

6. The pupae of DDT-resistant flies exhibit resistance to cyanide.

7. The effects of cyanide vary, depending on the developmental stage of the

pupae. A given concentration of the inhibitor produces different degrees of inhibition and mortality in pupae of different age. One of the possible explanations may be a change in the substrate being metabolized. Other possibilities are mentioned.

8. The possible mechanisms of resistance of pupae to cyanide are discussed. These may be: (a) a by-pass of the cytochrome system; (b) a difference in reversibility of enzyme-inhibitor complex or; (c) a detoxification of inhibitor. It is suggested that DDT-resistance may depend, in part, on similar factors.

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STUDIES OF THE DIGESTIVE TRACTS AND THE DIGESTIVE ENZYMES OF THE GOLDFISH, CARASSIUS AURATUS (LINNAEUS), AND THE LARGEMOUTH BLACK BASS, MICROPTERUS SALMOIDES (LACÉPÈDE) [1]

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A study of largemouth black bass, *Micropterus salmoides* (Lacépède), a carnivorous species, and goldfish, *Carassius auratus* (Linnaeus), an herbivorous species, was undertaken to determine the digestive enzymes secreted by the various regions of the digestive tracts and the associated glands, and to find the sites of their action. This study not only determined the type of foods that can be utilized, but also gave an idea of the relationship of digestive enzymes to different types of alimentary structures.

Previous Studies

Insofar as is known to the writer, no attempt has ever been made to study the digestive enzymes in goldfish and largemouth black bass. Markus (1932), however, determined the extent to which temperature changes influence food consumption in largemouth black bass.

A history of early investigations of digestion in fishes was given by Sullivan (1908). The earliest critical work mentioned was done by Spallanzani in 1783.

Sullivan studied the physiology of the digestive tract of elasmobranchs and found that the mucous membrane of the oesophagus was without glands; the stomachic sac secreted pepsin and hydrochloric acid; the gastric juice did not digest chitin; the mid-intestine and spiral valve had no digestive activity, but activated the pancreas, the latter having the greatest activating power; the pancreas secreted trypsinogen as a rule, but also secreted trypsin and in addition produced amylopsin and lipase; the rectal gland had no digestive activity.

Kingsley (1917) reported that gastric glands extended up into the oesophagus in sturgeons.

Bodansky and Rose (1922) made a preliminary study on digestion in certain elasmobranchs and teleosts and found that coagulated egg albumen was digested very slowly by the proteolytic enzymes of fishes. From extracts of the pyloric caeca of red snapper (*Lutjanus aya*), they obtained trypsin, pepsin, rennin, amylopsin, and lipase (weak). Invertase was present to a very slight extent; inulinase, maltase, and lactase were altogether absent.

Kenyon (1925) reviewed the works of earlier investigators and studied enzymes in bluegill, other fishes, amphibians, reptiles, and mammals. Erepsin was present in as great abundance in the intestinal mucosa of fishes, amphibians, and reptiles

[1] A thesis submitted to the Graduate Faculty of the Alabama Polytechnic Institute in partial fulfillment of the requirements for the degree of Master of Science in Fish Management.

as in the intestinal mucosa of dog; coagulated egg albumen was apparently digested equally well by the enzymes of fishes, amphibians, reptiles, and mammals; amylase was generally present in very small quantity in extracts of mucosa from the entire alimentary tract of fishes, amphibians, and reptiles. Invertase was found to occur in the intestinal mucosa of the bluegill.

Babkin (1927) found that extract of the intestinal mucous membrane of a top-minnow (*Fundulus heteroclitus*), a fish without a stomach, contained amylase, lipase, enterokinase, and erepsin. The bile from the gall bladder contained amylase, protrypsin, and prosteapsin.

Vonk (1927) found amylase and maltase in the pancreas of carp (*Cyprinus carpio*), pike (sp.), and dogfish (sp.).

MacKay (1929) studied the digestive system of the eelpout (*Zoarces anguillaris*), and found a strong lipase, a weak amylase, and a very weak protease in the mucous membrane of the stomach. A stronger amylase, a weaker lipase, and no protease were present in a mucous membrane of the duodenum. The juice taken from the stomach and duodenum showed a marked proteolytic action as well as an amylolytic and lipolytic action.

Chesley (1934) studied the concentrations of proteases, amylase, and lipase in certain marine fishes. He found that the digestive enzymes paralleled, in quantity, the general activity of the fish; this was especially true of trypsin but not of pepsin; lipase was the least abundant in the fishes that store large amounts of fat. The compact pancreas was the center of enzyme production, while the diffused pancreas produced only a small portion of the enzymes poured into the enteron. When the pancreas was not highly organized, the pyloric caeca seemed to supplant it partially in enzyme secretion.

Battle (1935) studied the digestive enzymes in the herring (*Clupea harengus*), and found pepsin in the extract of the mucosa of the pyloric caeca. Presence of weak lipolytic and amylolytic ferments was indicated in the gastric juice, and strong ones in extracts from the pyloric, caecal and intestinal mucosae.

Bayliss (1935) studied digestion in plaice (*Pleuronectes platessa*), and found that the mucous membrane of the intestine contains trypsin and erepsin; the liver and the wall of the gall bladder were more potent sources of trypsin than was the intestine; the former contained, also, a cathepsin, and the latter an erepsin. The mucous membrane of the intestine, the wall of the gall bladder, and the liver all contained lipase and amylase; the liver was a far more potent source of lipase than either the gall bladder or the intestine.

Digestive Tracts and Associated Glands

The digestive tract of goldfish is an elongated, relatively undifferentiated tube, coiling repeatedly and filling up the greater part of the abdominal cavity. A true stomach, comparable to that of the largemouth black bass is absent, and its place is taken by an enlargement of the intestine, which for convenience is designated as the intestinal bulb. The digestive tract could thus be separated into *mouth, buccal cavity, pharynx, oesophagus, intestinal bulb, anterior intestine,* and *rectum* (Figs. 1 and 2). The exact boundaries of the divisions of the intestine are not well-marked, but are indicated either by variations in calibre, by changes in the character of their epithelial lining, or by valve-like structures at the junctions of the adjoining regions (Fig. 2).

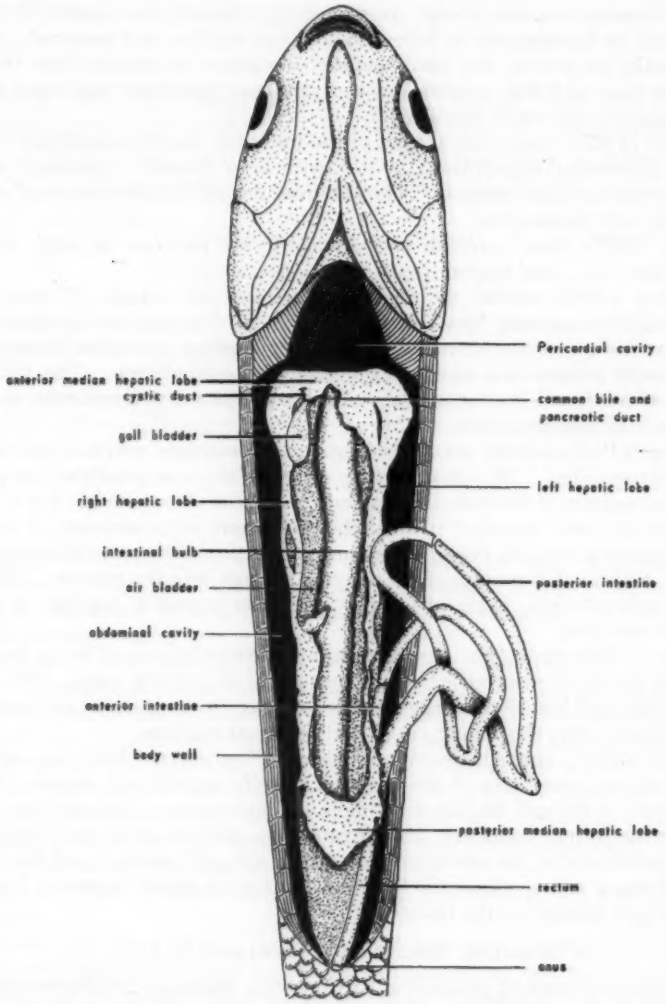

FIGURE 1. Ventral view of the goldfish with body wall and adipose tissue removed to show the viscera (breaks in the coil indicate portions of digestive tract omitted for the sake of brevity).

The *oesophagus* is a short tubular structure, dorsal to the pericardial cavity. The mucous membrane lining the wall is raised into 6 prominent longitudinal folds (Fig. 2). On entering the abdominal cavity, the digestive tract expands to form the intestinal bulb. Externally there is no well-marked indication of the passage of the oesophagus into the intestinal bulb except for an appreciable increase in the

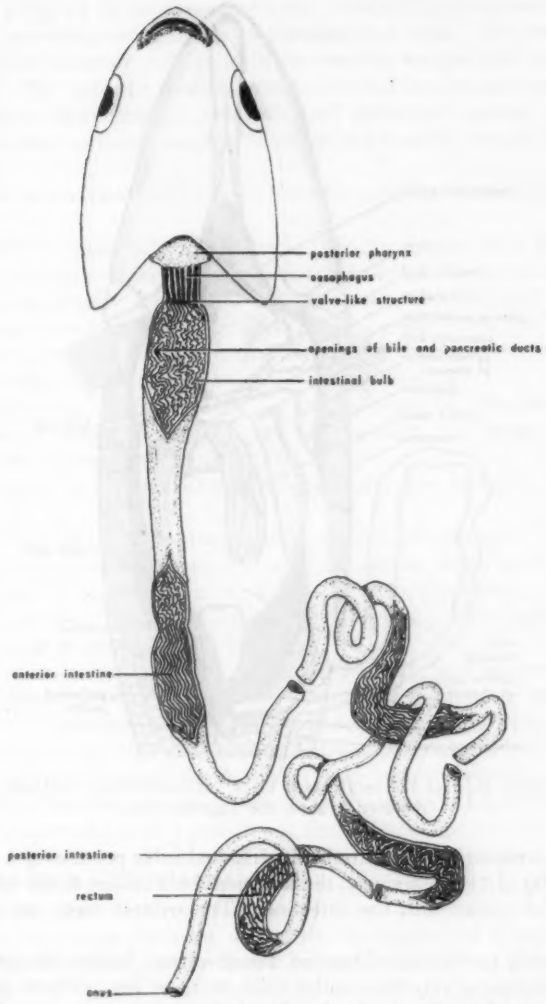

FIGURE 2. Dissection of digestive tract of goldfish to show the characteristics of the mucosal lining of different regions (breaks in the coil indicate portions of digestive tract omitted for the sake of brevity).

diameter of the latter. Internally the two are separated by a valve-like structure (Fig. 2).

The *intestinal bulb* lies dorsal to the coils of the intestine proper and ventral to the air bladder. The bulb is a more or less thickened straight tube, and extends almost to the posterior end of the abdominal cavity. The mucous membrane that

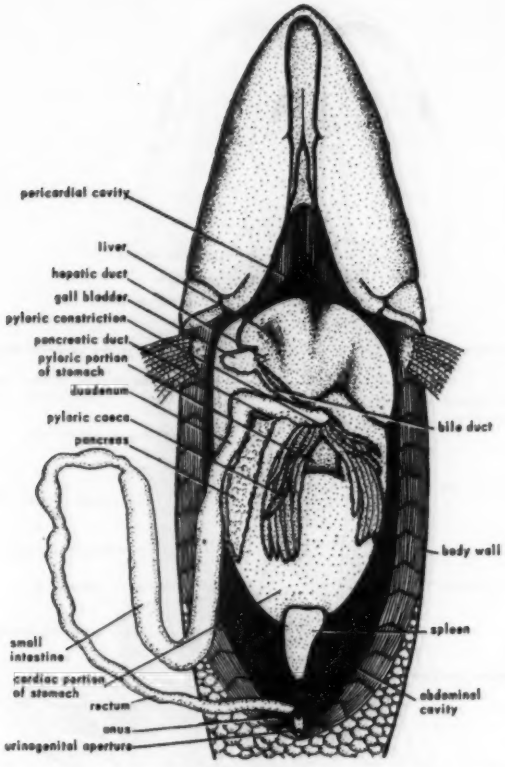

FIGURE 3. Ventral view of the largemouth black bass with body wall and adipose tissue removed to show the digestive tract.

lines the wall is raised into longitudinally disposed folds presenting a somewhat zig-zag pattern (Fig. 2). Posteriorly, the intestinal bulb curves to the left with a slight constriction and passes into the intestine. The pyloric caeca are absent in this species.

The remaining part of the elongated digestive tract behind the intestinal bulb is an elongated, comparatively thin-walled tube, more or less uniform in diameter and disposed into a number of intermixed coils, which fill the entire ventral portion of the abdominal cavity.

Two regions of the intestine proper are distinguished by the disposition of folds of the mucous membrane. The folds present a zig-zag pattern, which in the *anterior intestine* is composed of straight lines and angles, while in the *posterior intestine* it is produced by convoluted lines (Fig. 2).

The *rectum* is a relatively short tube, and is distinguished from the intestine by the presence of obliquely transverse folds in the mucous membrane. These anastamose at intervals (Fig. 2).

The *liver* (Fig. 1) is an elongated mass, consisting of narrow *right* and *left hepatic lobes*, extending on each side of the intestinal bulb. The two lobes join at their anterior and posterior ends to form the *anterior median* and *posterior median hepatic lobes*. The anterior median lobe is attached to the posterior face of the transverse septum separating the pericardial and abdominal cavities. The posterior median lobe is more or less crescentic in shape and extends beyond the limit of the bulb.

Wedged in between the right lobe of the liver and the intestinal bulb is the *gall bladder*.

The pancreas is apparently absent but is probably present in a diffuse mass imbedded in the substance of the liver as was found from a histological study (Sarbahi, 1940) in a closely related species, the Indian carp or rohu (*Labeo rohita*). The liver and the pancreas form the so-called hepato-pancreas. The bile and pancreatic ducts open on the roof of the intestinal bulb a short distance behind the valve-like structure separating the bulb from the oesophagus (Fig. 2).

The digestive tract of largemouth black bass could be differentiated into the *mouth*, the *buccal cavity*, the *oesophagus*, the *stomach*, the *duodenum*, the *small intestine*, and the *rectum* (Fig. 3).

The *oesophagus* is a short tubular structure and lies dorsal to the pericardial cavity.

The *stomach* could be differentiated into a *cardiac* portion and a *pyloric* portion. The cardiac portion, ovoid in shape and highly muscular, when distended with food fills a greater part of the abdominal cavity. The pyloric portion, a short muscular tube, branches off from the mid-ventral surface of the cardiac portion and passes into the duodenum at the pyloric constriction.

The *duodenum* is comparatively narrow. The bile and pancreatic ducts and 21 to 29 digitiform tubules (*pyloric caeca*) open into its anterior end immediately behind the pyloric constriction. The duodenum continues posteriorly into a relatively broad and straight tube, the *small intestine*. The small intestine passes into the narrow thin-walled *rectum*, which opens to the exterior through the anus.

The *liver* is a massive structure. Its anterior margin is crescentic and the posterior margin is provided with a pair of notches. These notches incompletely mark the liver into three lobes, which could be called the *right*, *median*, and *left lobes*. The *gall bladder* lies dorsal to the median lobe in close proximity to the left lobe.

The *pancreas*, a narrow elongated mass of tissue underlying the cardiac portion of the stomach, runs along the inner wall of the duodenum and is held in position by a mesentery attached distally to the duodenum and proximally to the cardiac portion of the stomach.

Materials and Methods

Preparation of tissue extracts and extracts of juices from the lumen of tracts

Glycerol extracts were prepared from the tissues of hepato-pancreas, oesophagus, intestinal bulb, anterior intestine, and posterior intestine of goldfish. Similar extracts were prepared from the liver, pancreas, pyloric caeca, oesophagus, stomach, duodenum, and small intestine of largemouth black bass.

The juices and lumen content from the intestinal bulb, anterior intestine, and posterior intestine of goldfish and from the stomach, duodenum, and small intestine of largemouth black bass were tested to determine the site of action of the various enzymes. After preliminary analyses, extracts from 13 goldfish and 7 largemouth black bass were used to establish fully the presence or absence of each enzyme in question. Each analysis was repeated two or more times on each sample. The size of the goldfish ranged from 13.5 to 20.7 cm. in total length and from 31 to 96 grams in weight. The size of the largemouth black bass ranged from 28 to 31 cm. in total length and from 369 to 454 grams in weight.

Freshly killed fish were immediately dissected under distilled water. The hepato-pancreas in goldfish and the liver, pancreas, and pyloric caeca in largemouth black bass were cleared of the mesentery and surrounding adipose tissue. They were then removed and thoroughly washed in distilled water, and were placed in 50 per cent glycerine covered with toluene for immediate trituration.

The digestive tracts of both the fishes were then ligated at the commencement of the several divisions to prevent passage of the contents from one division into another while being cleaned of the mesentery, and surrounding adipose tissue. Subsequently they were thoroughly washed, removed, wiped dry with filter paper, and cut into the several divisions as previously indicated. Each division was split open by a longitudinal slit, its contents were removed, and the lining was thoroughly washed in distilled water. The contents and washings constituted the sample from that division. If the lumen of any region was empty, it was customary to wash it with distilled water and to analyze the rinsing for the enzymes.

Tissue extracts from the goldfish were obtained by using the oesophagus, intestinal bulb, anterior intestine, and posterior intestine. In the largemouth black bass, tissue extracts were obtained from the mucosae of the oesophagus, stomach, duodenum, and small intestine. These were removed by scraping with a knife. Tissues of entire divisions were triturated in a mortar containing 25 to 50 milliliters of 50 per cent glycerol. Glycerol suspensions of the hepato-pancreas of goldfish and of the liver, pancreas, and pyloric caeca of largemouth black bass were prepared in a similar manner.

Water extracts of the juices or rinsings from various regions of the tracts and the finely ground tissue suspensions in glycerine of the two fishes were kept separately in 50 milliliter Erlenmeyer flasks away from direct sunlight for extraction of enzymes. The extraction was carried out at a temperature ranging from 18 to 22° C., with intermittent mechanical shaking.

The extracts of juices were strained or filtered after 24 to 48 hours; tissue extracts were strained through several thicknesses of cheesecloth after four to eight days. The interval permitted the extractions of enzymes, if present, in sufficient quantities to permit their detection by standard chemical methods.

Toluene was used throughout as a preservative and was added in sufficient quantity to form a layer one-eighth inch in thickness over the extract.

Tests of each extract were run for pepsin, trypsin, rennin, amylase, maltase, lactase, invertase, and lipase. Control samples, which contained an equal quantity of the extract, were boiled to destroy the enzymes and then were given the same treatment as the test samples of each analysis.

Digestive Enzymes

Pepsin

In this work, pepsin was considered to be the proteolytic enzyme which under strongly acid conditions (pH = 3) hydrolyzes proteins into proteoses and peptones. The tests used were not capable of determining whether the enzyme was identical with mammalian pepsin.

Fibrin stained in carmin (Hawk, Oser and Summerson, 1947) or 1.0 per cent Amaranth solution (Swingle, 1928) was used as substrate.

To 2 milliliters of glycerol extract of each of the several tissues and to 2 milliliters of the water extracts of juices from lumina of the several divisions of tracts of the two fishes was added a drop of congo red indicator, which produced a red color. This indicated a pH value of approximately 5. Sufficient 0.2 *N* HCl was added to cause congo red to turn blue—a pH of approximately 3. A small piece of the colored fibrin was placed in each of the test and control samples. Then the samples were covered with toluene and incubated at room temperature. Digestion was indicated if gradual dissolution of fibrin occurred.

In goldfish, pepsin was found to be absent from the digestive tract and the associated glands, since the fibrin was unaffected even after several weeks of incubation in the extracts.

In largemouth black bass, however, pepsin was present in both the extract from the mucosa of the stomach and the extract of juice from the lumen of the stomach. The action was considered slow, for it took two days for the small piece of fibrin to be completely digested. This slow action possibly could be attributed to reduced secretory activity of the glands, associated with low rate of feeding of the largemouth black bass during the cold weather of February and March.

Trypsin

In this work, trypsin was assumed to be present if digestion of proteins occurred under strongly alkaline conditions (pH = 8.4). The tests used were incapable of determining whether this enzyme was identical with mammalian trypsin.

Fibrin stained in congo red (Hawk, Oser and Summerson, 1947) or 1.0 per cent Ruthenium Red solution (Swingle, 1928) was used as substrate.

To 2 milliliters of glycerol extract of each of the several tissues and to 2 milliliters of water extract of juices from lumina of the several divisions of the digestive tracts of the two fishes were added 2 milliliters of water and 2 drops of cresol red indicator. Sufficient 0.2 *N* sodium carbonate was added to bring the cresol red to a faint pink, a pH of approximately 8.4. Each test and control sample received a small piece of colored fibrin, was covered with toluene, and was incubated at room temperature. Digestion was indicated by the gradual dissolution of the piece of fibrin.

In goldfish, trypsin was found to be present in extracts from hepato-pancreas, intestinal bulb, anterior intestine, and posterior intestine; and also in extracts of juices from lumina of the intestinal bulb, the anterior intestine, and posterior intestine. This indicated that in the absence of pepsin, trypsin was the only active protein-hydrolyzing enzyme, and was secreted not only in the hepato-pancreas but also in almost the entire length of the digestive tract except the oesophagus and the

rectum. Tissue extracts digested the fibrin within five to seven days. The extract of juice from the lumen of the intestinal bulb digested fibrin within 18 hours. Extracts of juices from the lumen of the anterior intestine and from the lumen of the posterior intestine took 24 to 36 hours and four to seven days, respectively, to complete digestion of similar pieces of fibrin. The rapidity of tryptic action in the extract of juice from lumen of the intestinal bulb may be related to the fact that the secretions from both the hepato-pancreas and the mucosa of the intestinal bulb itself pour into the lumen of this part of the tract.

In largemouth black bass, trypsin was found to be present in extracts from pancreas, liver, and pyloric caeca, and in extracts of juices from lumina of the stomach, duodenum, and small intestine. The extracts of juices from lumina of stomach, duodenum, and small intestine were found to be very active, for the fibrin was completely digested within 18 to 72 hours; the greatest activity was found in the extract of juice from the lumen of the stomach, wherein the fibrin was digested within 18 hours. The period for complete digestion of similar pieces of fibrin in extracts from mucosae of stomach, duodenum, and intestine ranged from five to ten days.

Rennin

In the presence of this enzyme, the casein of milk is hydrolyzed into soluble paracasein. The soluble paracasein combines with the soluble calcium salts of milk and an insoluble curd of paracasein results.

Fresh milk was used as substrate. To 1 milliliter of glycerol extract of each of the several tissues and to 1 milliliter of water extract of the juices from lumina of the several divisions of the tracts of the two fishes were added 5 milliliters of fresh milk. Test and control samples were covered with toluene and incubated at room temperature. Action of the enzyme is indicated by curdling of milk.

In both the goldfish and the largemouth black bass, rennin was found to be absent in the digestive tracts and the associated glands, since there was no separation of curd even after several weeks of incubation of milk in the extracts.

Amylase

In the presence of this enzyme, starch is hydrolyzed into dextrin and ultimately into maltose. Presence or absence of starch is indicated by the iodine test and the presence of reducing sugar (maltose) by the Fluckiger test as outlined by Swingle (1928).

One per cent freshly prepared starch solution was used as substrate for the iodine test. To 2 milliliters of glycerol extract of each of the several tissues and to 2 milliliters of water extract of the juices from lumina of the several divisions of the tracts of the two fishes were added 5 milliliters of starch solution. Several drops of I_2-KI solution (0.5 per cent I_2 in 1.5 per cent KI solution) were added in each of the test and control samples. The samples were covered with toluene and incubated at room temperature. Digestion was indicated by the solution turning colorless. Loss of color was very often found to be due to absorption of iodine by the tissue suspension and not due to digestion of starch. Upon addition of a few drops of I_2-KI solution the color again developed. In such cases, in-

cubation was continued with frequent addition of drops of I_2-KI solution until no blue color developed, at which stage digestion of starch was complete.

In the Fluckiger test for reducing sugar, a drop of 20 per cent NaOH was mixed with about an equal quantity of powdered copper tartrate upon a microscope slide until the powder was dissolved. A drop of the incubated starch solution was added and then the slide was gently warmed. Presence of maltose was indicated by a red precipitate of copper.

In the goldfish, amylase was found to be present in tissue extracts from hepato-pancreas, oesophagus, intestinal bulb, anterior intestine, and posterior intestine; and in extracts of juices from lumina of the intestinal bulb, the anterior intestine, and the posterior intestine. This indicated that amylase, like trypsin, was secreted not only in hepato-pancreas but also in a major part of the mucosal lining of the digestive tract. Tissue extract from hepato-pancreas took 24 hours to digest the starch completely. The actions in the rest of the extracts were less rapid and quite variable. It took two to four days for the starch to become completely digested.

In the largemouth black bass, amylase was found to be absent in the digestive tract and associated glands.

Maltase

In the presence of this enzyme, maltose is hydrolyzed into glucose, a reducing sugar. Benedict's solution becomes filled with red, yellow, or green precipitate of cuprous oxide, depending upon the amount of sugar present. If no glucose is present the solution remains perfectly clear. A counter check was run by use of Barfoed's solution (Hawk, Oser and Summerson, 1947), which is also a copper reduction test. Since maltose is a reducing sugar, careful control of the heating times in both Benedict's and Barfoed's test was used to differentiate this sugar from glucose.

One per cent freshly prepared maltose solution in distilled water was used as substrate in both the tests. The distilled water was thoroughly boiled to eliminate carbon dioxide.

To 1 milliliter of glycerol extract of each of the several tissues and to 1 milliliter of water extract of the juices from lumina of the several divisions of the tracts of the two fishes were added 5 milliliters of maltose solution (Benedict's test). The test and control samples were covered with toluene and incubated at room temperature for 24 to 96 hours. After incubation the test and control samples were filtered. The filtrates were tested for glucose. To 5 milliliters of the Benedict's solution in a test tube, exactly 8 drops of the filtrate were added and mixed well. The mixture was placed in boiling water for three minutes and then allowed to cool. In the presence of glucose, a red, yellow, or green precipitate of cuprous oxide was formed.

To 5 milliliters of Barfoed's solution was added 1 milliliter of filtrate prepared as previously indicated (Barfoed's test). After mixing well it was boiled for 30 seconds and then allowed to cool. In the presence of glucose, a red, yellow, or green precipitate of cuprous oxide was formed.

In goldfish, maltase was found to be present in extracts from hepato-pancreas, oesophagus, intestinal bulb, anterior intestine, and posterior intestine; and in

extracts of juices from lumina of intestinal bulb, anterior intestine, and posterior intestine. This indicated that maltase, like trypsin and amylase, was secreted not only in hepato-pancreas but also in a major part of the mucosal lining of the digestive tract. The digestive action in extract from hepato-pancreas was relatively weak as compared to the action in extracts from the intestinal bulb, anterior intestine, and posterior intestine. This indicated that the role of hepato-pancreas in secretion of maltase was only secondary. The action of extracts from intestinal bulb, anterior intestine, and posterior intestine was variable, being the weakest in extracts from mucosa of the intestinal bulb and from the juice of its lumen.

In largemouth black bass, maltase, like amylase, was found to be absent in the digestive tract and the associated glands.

Lactase

In the presence of this enzyme, lactose is hydrolyzed into glucose and galactose. Benedict's and Barfoed's tests were run to determine presence of glucose. Since lactose is also a reducing sugar, careful control of the heating times was used to differentiate this sugar from glucose.

One per cent freshly prepared lactose solution in distilled water was used as substrate in both the tests.

In both the goldfish and the largemouth black bass, lactase was found to be absent in the digestive tracts and the associated glands.

Invertase

In the presence of this enzyme, sucrose is hydrolyzed into glucose and fructose, both reducing sugars. Benedict's, Barfoed's, and Fluckiger tests were run to determine the presence of the reducing sugars.

One per cent freshly prepared sucrose solution in distilled water was used as substrate in both the Benedict's and Barfoed's tests. The distilled water was boiled to eliminate carbon dioxide. In Fluckiger's test, extracts incubated in 15 per cent sucrose solution for 48 to 96 hours were used. Fructose reduces the copper instantly in the cold. Glucose reduces it upon warming.

In goldfish, invertase was found to be present in extracts from hepato-pancreas, oesophagus, intestinal bulb, anterior intestine, and posterior intestine; and in extracts of juices from lumina of the intestinal bulb, anterior intestine, and posterior intestine. This indicated that invertase, like amylase and maltase, was secreted not only in hepato-pancreas but also from a major part of the mucosal lining of the digestive tract.

In largemouth black bass, invertase, like amylase, maltase and lactase, was found to be absent in the digestive tract and the associated glands, since the extracts did not respond to any of the several tests run.

Lipase

In the presence of this enzyme, fats are hydrolyzed into fatty acids and glycerol.

Emulsified olive oil containing brom thymol blue indicator and whose pH had been adjusted to approximately 7.2 was used as substrate.

To 2 milliliters of glycerol extract of each of the several tissues and 2 milliliters of water extract of the juices from lumina of the several divisions of the tracts of the two fishes were added 2 milliliters of emulsified olive oil after the pH of the extracts had been adjusted to 7.2. The test and control samples were covered with toluene and incubated at room temperature. Digestion was indicated by a change of color of the emulsion from blue to greenish yellow or yellow caused by free fatty acids.

In goldfish, lipase was found to be present in extract from hepato-pancreas, and in extracts of juices from lumina of intestinal bulb and anterior intestine.

In largemouth black bass, lipase was found to be present in extracts of juices from the lumen of the duodenum and small intestine.

General Conclusions and Discussion

These investigations revealed that a correlation existed between the structures of the alimentary canal and the occurrence and distribution of digestive enzymes. In goldfish, where the hepato-pancreas is the only accessory secretory gland associated with the digestive tract, lipase was evidently of little importance and was found in extracts from the hepato-pancreas alone. Trypsin was the only protease found, and it occurred in extracts from hepato-pancreas, intestinal bulb,

Table I

The digestive enzymes in goldfish and largemouth black bass

Species	Secretory organ	Pepsin	Trypsin	Rennin	Amylase	Maltase	Lactase	Invertase	Lipase
Goldfish	Hepato-pancreas	−	+	−	+	+	−	+	+
	Oesophagus	−	−	−	+	+	−	+	−
	Intestinal bulb	−	+	−	+	+	−	+	−
	Anterior intestine	−	+	−	+	+	−	+	−
	Posterior intestine	−	+	−	+	+	−	+	−
Largemouth black bass	Liver	−	+	−	−	−	−	−	+
	Pancreas	−	+	−	−	−	−	−	−
	Pyloric caeca	−	+	−	−	−	−	−	+
	Oesophagus	−	−	−	−	−	−	−	−
	Stomach	+	−	−	−	−	−	−	−
	Duodenum	−	−	−	−	−	−	−	−
	Small intestine	−	−	−	−	−	−	−	−

Explanation: The + indicates a positive test for the enzyme. The − indicates a negative reaction for the enzyme.

anterior intestine, and posterior intestine. The carbohydrases, amylase, maltase and invertase were found in extracts not only from the hepato-pancreas, but also in tissue extracts from a major part of the length of the digestive tract, including the oesophagus (Table I); they were active in the lumen throughout almost the entire length of the tract (Table II).

The digestive activity of the oesophagus in fishes has been reported by only a few investigators (Sullivan, 1908; Kingsley, 1917). The presence of the three carbohydrases in extracts from oesophagus of goldfish is in conformity with earlier findings (Sarbahi, 1940) of highly complex racemose glands in the mucosa of the

oesophagus of the rohu (*Labeo rohita*), which belongs to the same family (Cyprinidae) as goldfish.

Analyses of extracts of the hepato-pancreas and oesophagus indicated that the former secretes enzymes acting on carbohydrates, proteins, and fats, while the latter secretes enzymes acting on carbohydrates. The great digestive power of the secretion from the hepato-pancreas and the assumption of digestive activity by the oseophagus is closely associated with the absence of a compact pancreas and a true stomach. In *Labeo rohita* with a similar type of digestive apparatus, it was found (Sarbahi, 1940) that typical gastric glands also were absent.

The wide distribution of carbohydrases as compared to the distribution of proteinases and lipase indicated that goldfish could utilize foods that included a

TABLE II

Site of action of enzymes secreted in goldfish and largemouth black bass

Species	Site of action	Pepsin	Trypsin	Rennin	Amylase	Maltase	Lactase	Invertase	Lipase
Goldfish	Intestinal bulb	−	+	−	+	+	−	+	+
	Anterior intestine	−	+	−	+	+	−	+	+
	Posterior intestine	−	+	−	+	+	−	+	−
Largemouth black bass	Stomach	+	+	−	−	−	−	−	−
	Duodenum	−	+	−	−	−	−	−	+
	Small intestine	−	+	−	−	−	−	−	+

Explanation: The + indicates a positive test for the enzyme. The − indicates a negative reaction for the enzyme.

large percentage of carbohydrates, a fair amount of proteins, and a small amount of fats. The food of goldfish is chiefly plant materials that are rich in carbohydrates and contain fair amounts of proteins.

Foods of plant origin, being low in concentrates as compared to foods of animal origin, have to be consumed in bulk to meet the body requirements. Consumption in bulk implies longer retention in the tract for complete digestion. This involves the presence of a comparatively long digestive tract, such as is found in goldfish.

In largemouth black bass, proteinases are evidently the chief enzymes. Pepsin was found in extracts from the stomach and trypsin in extracts from liver, pancreas, and pyloric caeca. Lipase was found in extracts from liver and pyloric caeca. None of the carbohydrases was found in extracts either from glands associated with the digestive tract or from the tract itself.

The oesophagus of largemouth black bass, unlike the oesophagus of goldfish, had no digestive activity. This is closely associated with the presence of a compact pancreas and a true stomach.

The largemouth bass has a shorter digestive tract than that of the goldfish but has additional sources of enzymes to expedite digestion.

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